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(54) Title: METHODS AND COMPOSITIONS FOR TREATING CELL PROLIFERATIVE DISORDERS

(57) Abstract

The invention relates to agents and pharmaceutical compositions containing agents that regulate cellular protein kinase activity. Such compositions, when administered to cells expressing activated *ras*, induce these cells to undergo apoptosis. These compositions and agents can be used to treat diseases associated with protein kinase activity including carcinomas, sarcomas, lymphosarcomas, leukemias and other diseases which correlate with increased protein kinase activity and especially protein kinase C activity. The invention also relates to methods for determining the efficacy of chemotherapeutic agents and to methods for characterizing tumors by screening for reduced protein kinase activity.

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METHODS AND COMPOSITIONS FOR TREATING CELL PROLIFERATIVE DISORDERS

Rights in the Invention

This invention was made with United States Government support under grant number CA50459, awarded by the National Cancer Institute of the National Institutes of Health, and the United States Government has certain rights in the invention.

Background of the Invention

1. Field of the Invention

The invention relates to the treatment of proliferative disorders by the administration of agents that induce apoptosis in cells expressing activated oncogenes. The invention also relates to these agents, to methods of screening for new agents effective against specific disorders and to methods for staging neoplastic disorders.

2. Description of the Background

Neoplastic cells isolated from animals are frequently clonal in origin. Many of these cells have descended from a single ancestral cell, the founder cell, which lost the ability to regulate its own proliferation. Proliferation in defiance of genetic restraints leads to invasion and colonization of territories reserved for non-neoplastic or otherwise normal cells.

Neoplasms which proliferate out of control and grow as single masses are benign. Benign tumors often can be successfully treated by surgical removal of the tumor mass. Neoplastic cells that invade other tissue are malignant. Such cells enter the bloodstream or lymphatic systems and form metastases which are particularly difficult to treat. In fact, many tumors which initially appear benign may already have formed as yet undetectable metastases. Invasion of proximal or distal tissues is characteristic of malignancies, however, uncontrolled proliferation is common to all neoplasms and other proliferative disorders.

Normal cells are genetically programmed to self destruct through

apoptosis (E. White, Genes & Development 7:2277-84, 1993). Uncontrolled proliferation typically indicates that genetic controls over the cell cycle have been reprogrammed (G.T. Williams et al., Cell 74:777-79, 1993). For example, uncontrolled proliferation can be induced *in vitro* by the over expression, mutation or activation of a single gene. Genes which have such pleiotropic effects are referred to as dominant oncogenes and, in fact, many spontaneous tumors isolated from humans have been found to contain active dominant oncogenes. Nucleic acid analysis of many of these tumor cells has shown that the most common of the activated oncogenes is *ras*.

Ras was first discovered as the oncogene of a rat sarcoma virus from which its name was derived. *ras* is also the most highly conserved of all known oncogenes, but is actually only one member of a family of *ras*-related oncogenes. This family includes the functional *ras* genes, Ha-*ras*, Ki-*ras* and N-*ras*, and two pseudo *ras* genes, which are located on human chromosome 6 and the human X chromosome.

Ras genes are functionally conserved across phylogenetic lines in addition to being conserved at the protein sequence level. The *ras* gene or a *ras* gene homolog has been found in nearly every eukaryotic organism studied including human, mouse, rat, chicken, fruit-fly, mollusk, slime mold, gold fish and yeast. In addition, Ras protein has been found in all tissue and cell lineages as well including in the developing embryo.

One common feature of its ubiquitous nature appears to be that *ras* activity is involved with the process of growth regulation (S.A. Moodie, Trends. Genet. 10:44-48, 1994). Over expression or mutation of certain dominant oncogenes such as *ras* causes expressing cells to divide uncontrollably (M.S. Marshall, Trends. Biochem. Sci. 18:250-54, 1993). *In vitro* studies have shown that *ras* expression is elevated 5 to 7 fold during the S-phase in tissue culture cells (H.R.L. Bourne et al., Nature 348:678-79, 1990). However,

unlike some oncogenes, *ras* expression does not always correlate with cell division. While rapidly dividing cells, such as regenerating rat liver, express *ras* at an elevated level, non-dividing tissue, such as brain, can also show elevated expression of *ras*.

Mutant *ras* genes are able to transform mammalian cells in culture. Microinjection of the oncogenic form of the human Ha-ras protein resulted in the rapid proliferation of formerly quiescent cells (J.R. Feramisco et al., Cell 38:109-17, 1984). Further, mammalian *ras* can support the growth of mutant yeast cells. Studies have also shown that *ras* proto-oncogene function is required for serum stimulated cell growth of NIH-3T3 cells (L.S. Mulcahy et al., Nature 313:241-43, 1985). Activated *ras* has also been found in tumors induced by chemical and physical agents.

Proteins encoded by the *ras* gene family, p21ras, bind guanine nucleotides, possess an intrinsic GTPase activity and alternate between active (GTP-bound) and inactive (GDP-bound) forms and serve as essential transducers of diverse physiological signals (J. Downward, Bioessays, 14:177-84 1992). For example, *ras* activity has been found to be essential for T cell antigen-receptor signal transduction (C.D. Baldari et al., J. Biol. Chem. 268:2693-98, 1993). Ras protein has also been implicated in mitogenic signaling pathways of Swiss mice 3T3 fibroblasts (R.B.S. Roden et al., Cell Growth Differ. 4:957-64, 1993). In T lymphocytes, p21*ras* activity is involved in both PKC-dependent and PKC-independent activation pathways (J. Downward et al., Immunol. Today 13:89-92, 1992). In addition, p21*ras* appears to be involved in the regulation of intracellular calcium oscillation (Y. Sugimoto et al., Exp. Cell Res. 203:230-35, 1992). In lymphocytes, a sustained increase in the cytosolic calcium level occurs for both cell activation (A.J. Weiss et al., Am. J. Physiol. 263:C1119-40, 1992) and apoptosis (R.C. Duke et al., FASEB J. 8:237-46, 1994). In primary culture cells, Ras protein induction of PKC activity has been shown to

delay eventual cell self destruction or apoptosis (V.A. Illeral et al., J. Immuno. 151: 2965-73, 1993). However, as primary cells are already committed to apoptosis, it is at best unclear what effect alterations of PKC activity would have on other cells. Moreover, no clear picture has evolved as to how these diverse functions interrelate through Ras protein activity.

The majority of transforming *ras* mutants in spontaneous human tumors, as shown by DNA sequencing, have point mutations in the *ras* coding regions. These mutations are localized to a few sites indicating that only a few positions in the *ras* gene, when mutated, will give rise to a protein capable of causing neoplastic transformation (J.L. Bos, Cancer Res. 49:4682-89, 1989). Such oncogenic mutations have been found in the 12th, 13th, 59th, 61st, 63rd, 116th and 119th codons of *ras* in naturally occurring tumors. A single point mutation in these sensitive sites appears to lead to activation and the induction of spontaneous tumors.

p21*ras* activity is thought necessary for mediating cell cycle progression, including G_0 to G_1, and G_1 or G_2 to M transitions and *ras* belongs to a family of G proteins important for the intracellular transmission of cell surface receptor signals (P.H. Howe et al., J. Biol. Chem. 268:21448-52, 1993). High levels of activated p21*ras* in the absence of cooperating oncogene expression, however, can result in G_2 cell cycle arrest (P. Hirakawa et al., Proc. Natl. Acad. Sci. USA 85:1519-23, 1988). The proto-oncogene *bcl-2* product can protect cells from apoptosis induced by certain biological or chemical reagents (M.I. Walton et al., Cancer Res. 53:1853-61, 1993). For example, studies have demonstrated that Bcl-2 functions in an anti-oxidant pathway to prevent apoptosis (D.M. Hockenbery et al., Cell 75:241-51, 1993). Bcl-2 also blocks chemotherapy-induced apoptosis in human leukemia cell line (T. Miyashita et al., Blood 81:151-57, 1993). Interestingly, Bcl-2 protein has been shown to associate with a human Ras-related protein, R-ras or p23 (M.J.

Fernandez-Sarabia et al., Nature 366:274-75, 1993).

Three known families of cell surface receptors, classified by their mechanism of action, exist in humans. Channel-linked receptors are neurotransmitter-gated ion channels that open and close briefly in response to neurotransmitter binding and thereby transiently alter the electrical excitability of the cell. Catalytic receptors are mainly tyrosine-specific protein kinases that directly phosphorylate target cell proteins on tyrosine residues. G-protein-linked receptors individually activate or inactivate plasma-membrane-bound enzymes or ion channels through GTP binding proteins, G proteins. Some G protein-linked receptors activates or inactivates adenylate cyclase while others activate a phosphoinositide specific phospholipase C which hydrolizes phosphatidylinositol biophosphate (PIP_2) to generate inositol triphosphate and diacylglycerol. Diacylglycerol activates protein kinase C which in turn phosphorylates various cell proteins. Protein kinase C is one of several serine-threonine kinases implicated in cell proliferation and differentiation.

When activated by diacylglycerol, protein kinase C transfers the terminal phosphate group from adenosine triphosphate to specific serine or threonine residues on target proteins. In many cells, protein kinase C is thought to phosphorylate and activate the plasma membrane sodium-hydrogen ion exchanger which controls intracellular pH. The highest concentration of protein kinase C is found in the brain, where protein kinase C phosphorylates ion channels in nerve cells and alters their excitability threshold. In some cells, activation of protein kinase C increases the transcription of specific genes. The promoter of at least some of these genes contain a common transcription enhancer sequence, TGANTCA, that is recognized by the gene regulatory protein, *jun*, whose activity is stimulated by protein kinase C activity.

Summary of the Invention

The present invention overcomes the problems and disadvantages

associated with current strategies and designs and provides new methods and compositions for the treatment and prevention of neoplastic diseases and other disorders of cell proliferation.

One embodiment of the invention is directed to pharmaceutical compositions comprising agents that induce apoptosis of proliferating cells by reducing the activity of a cellular protein kinase. Effective agents include calphostin C, chelerythrine chloride, H7, hexdecyl-methylglycerol, hexadecyl-acetylglycerol, hypericin, K252, RO318220, phloretin, sphingosine, bryostatin and staurosporin. Aberrantly proliferating cells which may be effectively treated include, for example, malignant human neoplastic cells or virally infected cells. Compositions may also contain an agent that regulates intracellular calcium concentrations.

Another embodiment of the invention is directed to pharmaceutical compositions comprising agents that regulate intracellular calcium concentrations. Effective pharmaceuticals include agents such as calcium ionophores. Calcium ionophores increase intracellular calcium concentrations by increasing calcium ion transport across cell membranes and, in proliferating cells, induce apoptosis. Effective calcium ionophores include thapsigargin, ionomycin and A23187.

Another embodiment of the invention is directed to methods for inducing apoptosis of proliferating cells. Agents that regulate protein kinase activity are administered to aberrantly proliferating cells that express an activated oncogene. Activated oncogenes may be endogenous or introduced by transfection with oncogenic sequences. Suppression of intracellular protein kinase activity induces cells to undergo apoptosis.

Another embodiment of the invention is directed to methods for inducing apoptosis of proliferating cells. Agents that regulate intracellular calcium concentrations are administered to cells to increase intracellular calcium

concentration. Increased intracellular calcium concentration induces cells expressing activated oncogenes to undergo apoptosis.

Another embodiment of the invention is directed to methods for treating disorders comprising the step of administering a therapeutically effective amount of a pharmaceutical agent that induces apoptosis of aberrantly proliferating cells. Disorders which can be treated include neoplasms and infections wherein the aberrant cells express activated oncogenes. A therapeutically effective amount of the agent is that amount which reduces intracellular protein kinase activity or increases calcium concentrations sufficiently to induce apoptosis.

Other embodiments and advantages of the invention are set forth, in part, in the description which follows and, also in part, will be obvious from this description or may be learned from the practice of the invention.

Description of the Drawings

Figure 1 Relative cell survival based upon DNA fragmentation of untransformed, activated *ras* transformed, *bcl-2* transformed, and activated *ras* and *bcl-2* transformed Jurkat cell lines.

Figure 2 Relative cell survival based upon DNA fragmentation of untransformed, activated *ras* transformed, *bcl-2* transformed, and activated *ras* and *bcl-2* transformed Jurkat cell lines after 24 hours of treatment with PMA.

Figure 3 Percent of DNA fragmentation of untransformed, activated *ras* transformed, *bcl-2* transformed, and activated *ras* and *bcl-2* transformed Jurkat cell lines at 48 hours after 24 hours of treatment with PMA.

Figure 4 Flow cytometric DNA profiles of untransformed, activated *ras* transformed, *bcl-2* transformed, and activated *ras* and *bcl-2* transformed Jurkat cell lines with and without 24 hours of PKC

treatment.

Figure 5 Metabolic labeling and co-immunoprecipitation of p21*ras* and p26*bcl-2*.

Figure 6 Immunoprecipitation and detection of p21*ras* and p26*bcl-2* protein using monoclonal antibodies.

Figure 7 RNA blot analysis of *ras* RNA levels of specific subclones of activate *ras* transformed Jurkat cells (PH1) following down regulation of PKC.

Figure 8 DNA fragmentation analysis of specific subclones of activate *ras* transformed Jurkat cells (PH1) following down regulation of PKC.

Figure 9 DNA fragmentation analysis of Jurkat cell and activate *ras* transformed Jurkat cells (PH1) following down regulation of PKC with PMA for 24 hours after cycloheximide (CHX) treatment for 6 hours.

Figure 10 DNA fragmentation analysis of Jurkat cell and activate *ras* transformed Jurkat cells (PH1) following down regulation of PKC with PMA for 24 hours after EGTA treatment for 6 hours.

Figure 11 Growth curve of Jurkat cell and activate *ras* transformed Jurkat cells (PH1) in the presence or absence of calcium ionophore.

Figure 12 DNA fragmentation analysis of Jurkat cell and activate *ras* transformed Jurkat cells (PH1) in calcium ionophore with or without the addition of calcium chelator.

Figure 13 DNA fragmentation of activated *ras* transformed Jurkat cells (PH1) during serum deprivation.

Description of the Invention

As embodied and broadly described herein, the present invention

is directed to agents, compositions and methods for the treatment of cell proliferative disorders and also to methods for typing and for screening for additional agents which may be useful for the treatment of such disorders.

One of the most difficult disorders to treat effectively and with consistency is cancer. Although thousands of chemical agents have been tested, few have proven to be specifically effective against neoplasia and there is still no broad-based therapy available. It has been discovered, quite surprisingly, that agents effective at reducing cellular protein kinase activity, and specifically protein kinase C (PKC) activity, selectively induce cells that express activated oncogenes to undergo apoptosis. Induction of apoptosis causes neoplastic cells to differentiate, loose their aggressive nature and die. Cells that express an activated oncogene can be selectively induced to undergo apoptosis upon suppression of cellular protein kinase activity. Normal, non-neoplastic cells and neoplastic cells that do not express activated oncogenes are unaffected by treatment.

As many cancers possess activated oncogene activity, and especially activated *ras* activity, agents that induce apoptosis of cells transformed by activated *ras* or other activated oncogenes can be effectively used in a wide variety of proliferative disorders as prophylaxis or therapy. In addition, agents which increase intracellular calcium concentrations also induce aberrantly proliferating cells to undergo apoptosis. Calcium regulatory agents may also be involved in the regulation of protein kinase activity as most of these protein kinases are calcium dependant.

One embodiment of the invention is directed to pharmaceutical agents that selectively induce apoptosis when administered to aberrantly proliferating cells that express an activated oncogene. As a large number of neoplastic cells express activated oncogenes, these agents represent a broadly applicable new approach to treating a wide variety of mammalian disorders

including cancer, infection-induced malignancies, hyperplasia, hypertrophy and most any undesirable cell proliferation.

Agents which regulate protein kinase activity either by increasing or decreasing protein kinase activity include phorbols, acylglycerols and nucleic acids. Phorbol esters, for example, first stimulate and subsequently suppress protein kinase activity. The phorbol may be a phorbol ester such as phorbol myristyl acetate, phorbol butyrate, phorbol dibutyrate, deoxyphorbol phenylacetate, tetradecanoyl phorbol acetate, phorbol diacetate, phorbol didecanoate, alpha-phorbol didecanoate and deoxyphorbol isobutyrate. Other agents which suppress protein kinase activity, and specifically PKC activity, include, for example, staurosporine, sphingosine, hypericin, bryostatin, K252, phloretin, RO318220, H7, calphostin C, chelerythrine chloride and acylglycerol, and derivatives and functional equivalents of these agents such as monoacylglycerol, diacylglycerol, 1-oleoyl-2-acetylglycerol, hexadecyl-methylglycerol and hexadecyl-acetylglycerol, and diaoctanoylglycerol.

Agents are preferably safe and effective at useful dosages. Although side effects may occur, safe compositions are those wherein the benefits achieved from their use outweigh disadvantages attributable to adverse side effects. Unwanted side effects include nausea, vomiting, hepatic or renal damage or failure, hypersensitivity, allergic reactions, cardiovascular problems, gastrointestinal disturbances, seizures and other central nervous system difficulties, fever, bleeding or hemorrhaging, serum abnormalities and respiratory difficulties.

Most agents are biologically safe at effective doses whereas others may be chemically modified to remove or to add chemical moieties without hindering efficacy. Most agents are safe at effective doses or could be effectively administered at doses which are well-below toxic or mutagenic levels. Transient administration may also prevent or reduce negative consequences

attributed to side effects. Therapeutically effective pharmaceuticals may be created by modifying effective agents so that after introduction into the patient, these agents metabolize into active forms which have the desired effect on the patient. Effective pharmaceuticals may also be created which metabolize in a timed-release fashion allowing for a minimal number of introductions which are efficacious for longer periods of time. Combinations of agents can also produce useful new agents from the interaction of the combination. Such agents may also produce a synergistic effect when used in combination with other known agents.

Compositions can be prepared in solution as a dispersion, mixture, liquid, spray, capsule or as a dry solid such as a powder or pill, as appropriate or desired. Solid forms may be processed into tablets or capsules or mixed or dissolved with a liquid such as water, alcohol, saline or other salt solutions, glycerol, saccharides or polysaccharide, oil or a relatively inert solid or liquid. Liquids administered orally may include flavoring agents such as mint, cherry, guava, citrus, cinnamon, orange, mango, or mixed fruit flavors to increase palatability. Pills, capsules or tablets administered orally may also include flavoring agents. Additionally, compositions may further comprise agents to increase shelf-life, such as buffers, preservatives, anti-oxidants and other components necessary and suitable for manufacture and distribution of the composition. Compositions further comprise a pharmaceutically acceptable carrier. Carriers are chemical or multi-chemical compounds that do not significantly alter or effect the active ingredients of the compositions. Examples include water, alcohols such as glycerol and polyethylene glycol, glycerin, oils, salts such as sodium, potassium, magnesium and ammonium, fatty acids, saccharides or polysaccharides. Carriers may be single substances or chemical or physical combinations of these substances.

Pharmaceutical compositions may also comprise other agents that regulate intracellular calcium concentrations. Calcium regulators may be, for

example, calcium ionophores which increase transport of calcium ions across cell membranes. As pharmaceuticals, these agents alter intracellular calcium levels and induce apoptosis. Calcium ionophores that increase intracellular calcium ion levels include A23187, thapsigargin and ionomycin. These agents are also effective at regulating the activity of protein kinase and specifically protein kinase C. Such agents may be administered before, concomitant with or after the administration of other agents which regulate protein kinase activity.

Additional agents which are effective at selectively inducing apoptosis include anti-sense nucleic acids such as DNA, RNA or PNA oligonucleotides comprising a sequence complementary to a translated or transcribed sequence of the protein kinase gene. Anti-sense technology exploits the specificity of hybridization reactions between two complementary nucleic acid chains to prevent the expression of that gene. For example, transcription can be inhibited by introducing a single-stranded nucleic acid, complementary to a messenger RNA of the protein kinase or another gene which positively effects kinase activity or expression. Nucleic acids introduced will hybridize, for example, to protein kinase mRNA in the cytoplasm and thereby inhibit translation and subsequent expression of the protein kinase gene. Another method to inhibit protein kinase expression is to introduce anti-sense nucleic acid to protein kinase mRNA or other exogenous oligonucleotides to neoplastic cells. Anti-sense oligonucleotides may be introduced with viral or other vectors which infect or are transfected into target cells. Anti-sense nucleic acid may be complementary to the coding region, the 5'- and 3'-non-coding regions, or to splice sites of protein kinase mRNA. The anti-sense nucleic acid may also comprise a transcription template, a double stranded DNA with an RNA transcription promoter linked to an anti-sense gene. After this transcription template enters a cell, it can direct the synthesis of anti-sense RNA. The anti-sense RNA than binds to and inactivates the mRNA of the particular protein

kinase causing the cells to undergo apoptosis.

Apoptosis can be described as an intracellular mechanism of programmed cell death. It can be characterized by, for example, chromatin condensation, decreased cell volume, untethering of cell membrane from cytoskeleton (zeiosis), quiescence, decreased rate of mitosis and cleavage of DNA. Although the mechanism appears to be largely intracellular, intercellular signaling may be involved to some degree. In contrast, simple necrosis or tissue trauma typically involves cellular cytoplasmic and plasma membrane damage, osmotic swelling and cell lysis. Although an apoptotic cell may have one or more apoptotic characteristic symptoms before death, absolute determinations can be made with observations in comparison to control cells.

Apoptosis can be measured by observing certain of these parameters such as the degree of DNA fragmentation. For example, in apoptotic cells as compared to control samples, nucleic acid fragmentation is generally greater than about two fold, preferably greater than about four fold, more preferably greater than about ten fold, and even more preferably greater than about twenty fold. Other parameters which can be used to measure the degree of apoptosis include the rate of mitosis, the amount of chromatin condensation, alterations in cell size or volume or simply decreased metabolism or metabolic rate as measured by the production of metabolic products such as acids or enzymes.

Cells that express an activated oncogene are typically abnormal having irregular margins or membranes, unusual mitotic activity, DNA damage or mutations, increased size or volume, unique gene expression patterns, unusual chromatin structure and sub-cellular alterations such as substantially increased numbers of mitochondria. Such abnormal cells demonstrate uncontrolled proliferation which can cause a variety of benign and malignant neoplasms. While it is normal for different cells to divide at different rates in a body,

aberrant uncontrolled proliferation is the ability of a cell to divide significantly more often than other cells of the same linage. In the adult, cells such as nerve cells are mostly incapable of further division once development has ceased. Other cells such as liver, thyroid and other glandular cells, once mature seldom divide, but are capable of rapid division on demand such as, for example, after partial destruction of the liver. Still others such as skin cells, bone marrow cells and the circulating cells divide continually throughout life to replace damaged or retired cells of the system. Thus, aberrant uncontrolled proliferation encompasses cell division in a normally non-dividing cell to significantly increased division in a normally dividing cell. While some neoplastic cells retain many features of the specific cell type from which they are derived, many neoplastic cells develop characteristics commonly associated with neoplasms. These characteristics include invasion of the basal lamina, the establishment of metastases, loss of differentiation characteristics, abnormal variability in size and shape, and hypertrophy and hyperplasia.

Many characteristics of neoplastic cells, not apparent from an *in situ* examination, but are readily evident *in vitro*. *In vitro* characteristics include a lack of contact inhibition, growth in soft agar, mutation or over expression of a dominant oncogene, low or no expression of tumor suppression genes, homozygosity of recessive or mutated oncogenes, unstable karyotypes, aneuploidy, anchorage independent growth, chromosomal translocations, double minute chromosomes and defects in nucleic acid repair, and unusual or abnormal transcription or replication, or combinations of these *in vitro* or other *in vitro* traits.

Many human neoplasms contain cells which express activated oncogenes. An activated oncogene is a gene with a mutation which cause a cell to become neoplastic. The mutation may be of any form in both the coding and non-coding, regions of the gene. Non coding mutations may occur at the 5'- or

3'-ends of the gene. These mutations include point mutations, frame shift mutations, amplifications, translocations, insertions, deletions, promoter mutations, enhancer mutations, translocations and capture by virus. Oncogene activations may include more than one mutation.

Oncogenes families which contain dominate oncogene mutations include members of the *ras* family of genes such as Ha-*ras*, Ki-*ras* and N-*ras* which encode p21*ras* proteins and are associated with some of the most common forms of human neoplasms. Other oncogenes include *abl, crk, dsi, erb, ets, evi, fim, fes/fps, fgr, fms, fos, gli, int, jun, kit, lck, mas, mis, mil/raf, mos, myb, myc, neu, pim, rel, ros, seq, sis, ski, spi, src, trk* and *yes*. Mutated forms of p53 and related proteins are also considered herein to be expressed from activated oncogenes.

Another embodiment of the invention is directed to a method for inducing a population of aberrantly proliferating cells to undergo apoptosis by treatment with agents that regulate protein kinase activity. Cell populations which are treatable can be identified by the expression of activated oncogenes such as, for example, members of the *ras, myc, fos, jun, mos* and *src* family of genes. Expression can be from inherent sequences of the cells or from transfected oncogene sequences.

Activated oncogenes can be detected by genetic analysis of the cells. Cell samples can be obtained using a wide variety of invasive or non-invasive techniques. For example, surgical biopsy can be used to remove samples of tissue to make observations, to purify nucleic acid, if necessary, for RFLP (restriction-fragment length polymorphism), PCR (polymerase chain reaction) or direct DNA or RNA sequencing, or to culture. In addition, it is well-known that many tumors shed transformed cells into the surrounding areas around the tumor. Such cells can be easily collected and, thereafter, analyzed with minimal risks and discomfort for the patient. Confined areas which may

contain such cells can be sampled by, for example, needle aspiration. Samples of tissue or fluid can be obtained from various areas of the body and directly analyzed for genetic mutations such as evidence of activated forms of *ras*. For example, samples of pancreatic fluid will identify treatable forms of pancreatic cancers and stool samples will identify treatable bowel cancers. Other cancers which can be as easily identified include prostate, vaginal, brain, lung, bone marrow and tumors of various organs.

Although mutations including activating mutations may be inherent to the cell, many activating mutations, and consequently neoplasia, may be caused by exposure to chemical mutagens such as cigarette smoke and asbestos, by exposure to electromagnetic radiation such as sunlight and atomic radiation and by exposure to pathogens. Pathogens suspected of inducing neoplastic transformations directly or indirectly include human immunodeficiency virus (HIV), Epstein-Barr virus and other herpes viruses (HZV, HSV-1, HSV-2), hepatitis virus (types A, B, C, etc.), human T cell leukemia virus (HTLV-I, HTLV-II) and other retroviruses, human papilloma and polyoma viruses and many others.

While the relationship between aberrant proliferation and specific genetic mutations is not completely understood, the interrelation of protein kinase activity is even less clear. Protein kinases are enzymes which transfers the 2-phosphoryl group of adenosine triphosphate (ATP) to some nucleophilic acceptor protein. While ATP is the general phosphate donor, other purine or pyrimidine triphosphates such as guanosine triphosphate, uridine triphosphate, or cytidine triphosphate may also serve as phosphate donors. Protein kinases that are important cellular regulators of growth generally phosphorylates proteins on serines, threonines and tyrosines. These kinases include the serine/threonine-specific protein kinases, the serine-specific protein kinases, the tyrosine-specific protein kinases, the cAMP-dependent kinases (A-kinases), the cGMP-dependent

kinases (G-kinases), phosphorylase kinases and protein kinase C. Protein kinase C may have a direct connection with *ras* proteins as both products participate in the G-protein signal transduction pathway.

Disorders which can be treated include neoplastic and other disorders which contain cells that expresses activated oncogenes such as, for example, *ras* or an activated *ras*-like oncoprotein. Neoplasms which express activated *ras* include malignancies, pre-malignancies or diseases that result in a relatively autonomous growth of cells. Some specific neoplastic disorders which are prophylactically or therapeutically treatable include small cell lung cancers and other lung cancers, rhabdomyosarcomas, choriocarcinomas, glioblastoma multiformas (brain tumors), bowel and gastric carcinomas, leukemias, ovarian cancers, cervical cancers, breast cancer, pancreatic cancer, prostate cancers, osteosarcomas or cancers which have metastasized. Diseases of the immune system which are treatable by these compositions include the non-Hodgkin's lymphomas including the follicular lymphomas, Burkitt's lymphoma, adult T-cell leukemias and lymphomas, hairy-cell leukemia, acute myelogenous, lymphoblastic or other leukemias, chronic myelogenous leukemia, and myelodysplastic syndromes. Additional neoplastic diseases treatable by the compositions of this invention include virally-induced cancers wherein the viral agent is EBV, HPV, HTLV-1 or HBV. Other types of treatable cancers include breast cell carcinomas, melanomas and hematologic melanomas, ovarian cancers, pancreatic cancers, liver cancers, stomach cancers, colon cancers, bone cancers, squamous cell carcinomas, neurofibromas, testicular cell carcinomas and adenocarcinomas.

The patient to be treated may be any mammal such as a human including adults, children, infants and even a fetus. Treatments may be directly or indirectly administered to the patient. Direct administration may be by, for example, oral, parenteral, sublingual, rectal, pulmonary absorption or topical

application. Parenteral administration may be by intravenous injection, subcutaneous injection, intramuscular injection, intra-arterial injection, intrathecal injection, intra peritoneal injection or direct injection or other administration to one or more specific sites. Injectable forms of administration are sometimes preferred for maximal effect in, for example, bone marrow. When long term administration by injection is necessary, venous access devices such as medi-ports, in-dwelling catheters, or automatic pumping mechanisms are also preferred wherein direct and immediate access is provided to the arteries in and around the heart and other major organs and organ systems. Indirect administration is performed, for example, by administering the composition to cells *ex vivo* and subsequently introducing the treated cells to the patient. The cells may be obtained from the patient to be treated or from a genetically related or unrelated patient. Related patients offer some advantage by lowering the immunogenic response to the cells to be introduced. For example, using techniques of antigen matching, immunologically compatible donors can be identified and utilized.

Compositions may be administered as a bolus injection or spray, or administered sequentially over time (episodically) such as every two, four, six or eight hours, every day or every other day. Compositions may also be administered in a timed-release fashion such as by using slow-release resins and other timed or delayed release materials and devices. Orally active compositions are more preferred as oral administration is usually the safest, most convenient and economical mode of drug delivery. Oral administration is usually disadvantageous because compositions are poorly absorbed through the gastrointestinal lining. Agents which are poorly absorbed tend to be highly polar. Consequently, agents which are effective, as described herein, may be made orally bioavailable by reducing or eliminating their polarity. This can often be accomplished by formulating a composition with a complimentary

reagent which neutralizes its polarity, or by modifying the agent with a neutralizing chemical group. Oral bioavailability is also a problem because drugs are exposed to the extremes of gastric pH and gastric enzymes. These problems can be overcome in a similar manner by modifying the molecular structure to withstand very low pH conditions and resist the enzymes of the gastric mucosa such as by neutralizing an ionic group, by covalently bonding an ionic interaction, or by stabilizing or removing a disulfide bond or other relatively labile bond.

In addition, cells may be isolated from a patient and treated before returning the cell to the same or a different patient. This invention may be used, for example, in a bone marrow transplantation to treat a neoplastic disorder. The bone marrow of a patient is first removed and the cells are treated with the agents of this invention. After the patient is treated for the cell proliferative disorder, the treated cells are reintroduced into the patient. In some forms of neoplastic disease treatment, bone marrow cells are removed from a patient before chemotherapy and returned to the patient after chemotherapy. While the bone marrow cells are outside the patient, they are often sorted to remove neoplastic or pre-neoplastic cells. The bone marrow cells may be treated with the compositions of this invention *ex-vivo* before their return into the body. This treatment of this invention may also be used to treat bone marrow cells when the donor and the recipient are different individuals.

Another embodiment of the invention is directed to the administration of protein kinase suppressor agents which, in combination with other chemotherapeutic agents, maximize the effect of the compositions in an additive or synergistic manner. Other antineoplastic agents which can be administered with the agents of the invention include differentiating agents such as the butyrates (*e.g.* sodium butyrate, isobutyramide, lysine or arginine butyrate) and retinoic acid, alkylating agents such as alkyl sulfonates, aziridine,

ethylenimines and methylmelamines, nitrogen mustards, nitrosoureas, antibiotics, antivirals including ganciclovir, antimetabolites such as folic acid analogs, purine analogs, pyrimidine analogs, hormones such as androgens, antiadrenals, antiandrogens, antiestrogens, estrogens, luteinizing hormone and releasing hormone analogs and progestogen. In addition to chemotherapeutic agents, the antineoplastic agents of the invention may also be administered in conjunction with cytokines such as tumor necrosis factor (TNF), the interleukins (IL-1, IL-2, IL-3, IL-4, IL-5, etc.), the interferons (IFN-α, -β, -γ) and growth factors. Therapies using combinations of these agents would be safe and effective against malignancies and other forms of cancer. Combinations of therapies may also be effective in inducing regression or elimination of a tumor or some other form of cancer such as compositions of the invention plus radiation therapy, toxin or drug conjugated antibody therapy using monoclonal or polyclonal antibodies directed against the transformed cells, gene therapy or specific anti-sense therapy. Effects may be additive, logarithmic, or synergistic, and methods involving combinations of therapies may be simultaneous protocols, intermittent protocols or protocols which are empirically determined.

Another embodiment of the invention is directed to a method for screening for agents for their ability to induce apoptosis. These agents will be useful for the treatment of neoplastic and other cell proliferative disorders. Prospective agents may be tested by administering the agent to a cell line and assaying the intracellular protein kinase activity or calcium concentration of the cell line. Alternatively the agent may be administered to a cell line comprising, for example, activated *ras* and the cell line monitored for apoptosis. Agents which initially stimulate, but subsequently suppress protein kinase activity may be useful for treatment. Agents which enhance protein kinase activity may also be used to, for example, temporarily stimulate and subsequently inhibit protein kinase activity.

Another embodiment of the invention is directed to a method of typing neoplastic cells or potentially neoplastic cells of a patient, or of typing neoplastic cell lines in culture. Neoplastic cells may be isolated from a surgically removed a biopsy or other specimen, an established cell line, a virally infected cell line or a neoplasm which may be propagated in an immunocompromised host. Examples of immunocompromised hosts include nude mice, human immunodeficiency virus infected humans and animals. Nearly neoplastic cells may be pre-neoplastic cells, hyperplastic cells or hypertrophic cells. Cells to be assayed are treated with an agent which transiently down regulates protein kinase activity and the degree of apoptosis is monitored. If apoptosis occurs, the cell will be diagnosed as having an activated oncogene such as a *ras* gene or an activated *ras* functional homolog. If the cell which shows apoptosis after treatment is not cancerous, there will be strong indication that it may be pre-neoplastic and appropriate treatment may be considered.

Another embodiment of the invention is directed to methods for the treatment of patients with a neoplastic or other cell proliferative disorder comprising the administration of one or more pharmaceutical compositions of the invention. Compositions to be administered contain a therapeutically effective amount of the effective agent. A therapeutical effective amount is that amount which induces apoptosis or, otherwise, has a beneficial effect to the patient by alleviating one or more symptoms of the disorder or simply reduce mortality. For example, a beneficial effect may be a decrease in pain, a decrease in duration, frequency or intensity of blast crises in a leukemia patient, decreased fatigue or an increased strength. Preferably, a therapeutic amount is that amount of agent that stimulates or enhances the apoptosis of neoplastic or pre-neoplastic cells. A plurality of PKC inhibitors can be administered to a patient in the course of a treatment.

Treatments to the patient may be therapeutic or prophylactic. Therapeutic treatment involves administration of one or more compositions of the invention to a patient. Pharmaceutical compositions may also be administered to patients prophylactically. A diagnosis of a neoplastic disorder is often rather difficult for many types of tissues. Many patients in the early stages of pre-neoplastic or neoplastic disease may have no symptoms. Furthermore, a neoplasia may be too small to be detectable with current technology. Some patients, such as former cancer patients and patients with disorders such as Beckwith-Wiedemann syndrome, Aniridia, neurofibromatosis, multiple endocrine neoplasm or human immunodeficiency virus infection show a high probably of future neoplastic disorders. These patient can benefit from regular preventive treatment by the methods of this invention. Administration can begin at birth and continue, if necessary, for life. The administration of protein kinase inhibitors to asymptomatic, but high risk patients may help in the destruction of pre-neoplastic or neoplastic cells. Both prophylactic and therapeutic uses are readily acceptable.

Another embodiment of the invention is directed to a method for regulating the expression of a protein kinase gene in a mammalian cell. Briefly, the cell is exposed to an effective amount of a agent of the invention. A normally expressed protein kinase gene of the cell, such as protein kinase C, is suppressed to reduced the expression of its product. An effective amount of the composition is that amount which decreases the extent or magnitude of kinase activity.

Another embodiment of the invention is directed to a method for down-regulating the proliferation of cells expressing an activated oncogene such as, for example, activated *ras*. Such cells would be induced to undergo apoptosis or simply die. As above, an effective amount of a composition of the invention is exposed to cells *ex vivo* or administered to cells *in vivo*. These cells

can be utilized to treat neoplastic disorders by administration to patients. For example, bone marrow removed from a patient may be treated with the compounds of this invention and returned to a patient. Bone marrow cells can be obtained from volunteers or the patients to be treated.

The following experiments are offered to illustrate embodiments of the invention, and should not be viewed as limiting the scope of the invention.

Examples

Example 1 Establishment of a *Ras* and bcl-2 Transformed Cell Line.

Jurkat cells (American Tissue Culture Collection; Rockville, MD) were cultured in Dulbecco modified Eagles medium (DMEM) containing 10% heat-inactivated new born calf serum (Hazelton Research Products, Inc.; Lenexa, KA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were stably transfected by electroporation with a v-Ha-*ras* expressing vector and a Geneticin marker to establish a *ras* transformed cell line. Jurkat cell lines transfected with the neomycin resistance gene were used as controls. Positive transformants were selected in the same growth medium plus 0.7 mg Geneticin (GIBCO/BRL; Grand Island, NY) per ml.

A retroviral expression vector containing *bcl-2* and hygromycin resistance gene was transfected into the amphotropic murine retroviral vector packaging line DAMP to establish a bcl-2 cell line. Introduction of the bcl-2 gene into Jurkat cells was performed by infecting Jurkat cells with the packaged retrovirus in the presence of polybrene at 40 μg/ml. Jurkat cells with stable integration of bcl-2 was selected with growth medium containing 200 μg/ml hygromycin.

To determine if the transfected oncogenes affected the growth characteristics of the transfected cell lines, the division times of different cell lines were measured in growth media with and without phorbol esters. Cells (1 x 10^6 cells/ml) were incubated in 500 nM of phorbol myristyl acetate (PMA) for 24 hours, washed two times with PBS and seeded into 60 mm petri dishes (0.25 x 10^6 cell/ml, 5 dishes for each point). Viable cells were counted at daily intervals after staining with trypan blue dye.

Example 2 Cell Viability Determined from DNA Fragmentation.

Cell viability was monitored by cytometric analysis performed with a FACScan and the Cell-Fit software program (Becton Dickenson; Mountain View, CA). Briefly, after down-regulation of PKC and in the presence of chelators, protein synthesis inhibitors, or other reagents, 0.5 x 10^6 cells were washed with PBS twice, and resuspended in 1 ml of 1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide. Permeablized cells were stored in the dark at 4°C overnight before DNA profile or DNA fragmentation analysis.

Example 3 Analysis of Transformed Cell Lines.

In vivo phosphorylation of Bcl-2 protein: Cells (2 x 10^7) were prepared for phosphorylation by growth in phosphate free medium for 6 hours containing 2.5 mCi of [^{32}P]PO_4. Both normal cells and cell grown under 500 nM of PMA were phosphorylated. To isolate phosphorylated Bcl-2 protein, cell lysates were normalized for protein and incubated with an anti-human Bcl-2 antibody (Pharmingen; San Diego, CA). Immunocomplexes containing Bcl-2 were coupled to protein A and washed 5 times with 1 ml of lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 100 mM $NaCl_2$, 1 mg/ml BSA, 10 mM benzamindine, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor). Immunoprecipitates were analyzed by SDS-PAGE. Bcl-2 was detected after electrophoresis by autoradiography of the dried gel.

Immunoprecipitation and western blot: Cells (2 x 10^7) were washed twice in PBS in preparation for immunoprecipitation. Soluble protein were extracted by treating the cells with 1% Triton X-100, 50 mM HEPES, pH 7.4, 100 mM $NaCl_2$, 1 mg/ml BSA, 10 mM benzamindine, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor. After removal of the insoluble cell debris by centrifugation at 20,000 x g for 5 minutes at 4°C, the supernatant fraction was adjusted to 0.5 M NaCl, 0.5% deoxycholate and 0.05% sodium dodecyl sulphate. For each sample, a duplicate cell lysate preparation was made for immunoprecipitation and Western Blot experiments. 100 μg of cell lysate were used for each immunoprecipitation with either pan-*ras* antibody (Oncogene Science), or anti-human Bcl-2 antibody (DAKO; Malvern, PA). Lysates were co-incubated with antibodies for two hours, coupled to protein A and washed five times with 1 ml of lysis buffer. Immunoprecipitates were analyzed by 12.5% SDS-PAGE. After electrophoresis, proteins were detected by immunoblotting with specific antibodies and developed with an anti-mouse immunoglobulin alkaline phosphatase agent (Oncogene Science).

Immunoblot and kinase assay: Cells (2 x 10^7), under normal condition or after down-regulation of PKC activity, were permeabilized with 1 ml of kinase buffer containing 5 μCi of [^{32}P]ATP for 10 minutes at 37°C. For inhibition experiments, 0.1 μM of staurosporin or 200 μM genistein was added to the kinase buffer. Proteins were extracted from lysed cells and analyzed by SDS-PAGE. After electrophoresis, gels either were subjected to autoradiography directly or to western blot analysis and subsequently to autoradiography.

[^{35}S]-metabolic labeling of cellular proteins: Cells (2 x 10^7) were labeled by growth in 50 μCi of [^{35}S]-Methionine and 50 μCi of [^{35}S]-Cysteine for 3 hours. Labeled proteins were immunoprecipitated with specific antibodies and separated by 12.5% SDS-PAGE. Gels were fixed with 10% glacial acetic acid

and 30% methanol for 30 minutes, treated with an enhancing solution and subjected to autoradiography.

RNA blot analysis of total cellular RNA: Total cellular RNA was isolated by guanidine thiocyanate phenol RNA extraction, quantified, separated by electrophoresis on formaldehyde agarose gels and transferred to nitrocellulose. Hybridizations were performed at 68°C with 6xSSC, 2x Denhardts and 0.1% SDS, and washed at 68°C in 0.2xSSC plus 0.1% SDS.

[^{32}P]-DNA probes: [^{32}P]-labeled DNA probes were made by the random oligonucleotide primer method. The actin probe consisted of a 0.7 kb *Pst* I fragment encoding murine β-actin. The *ras* probe consisted of a 700-kb *Pst* I to *Sac* I fragment of the Harvey Sarcoma proviral clone pH1.

Example 4 <u>Transfected Constructs have no Effect on Cell Survival</u>.

Jurkat cells were transformed with the constructs of Example 1 and their growth monitored to determine the effects of the various constructs on survival. Untransformed, activated *ras* transformed (pH1), *bcl-2* transformed and activated *ras* and *bcl-2* transformed cells were monitored and their growth plotted (Figure 1). All four cell types show similar division times and survival rates. The transformation of the construct and the expression of the transgene appear to have no effect on survival and growth of cells under routine *in vitro* culture.

Example 5 <u>Cell Lines Expressing *ras* are Induced into Apoptosis by Phorbol Esters</u>.

Untransfected and activated *ras* containing Jurkat cells were exposed to 500 nM of phorbol ester (PMA) for 24 hours and then returned to culture with normal growth medium in the absence of PMA. Growth properties were compared to control cells cultured under the same conditions, but without PMA treatment. Surviving cells were enumerated at daily intervals based on trypan blue exclusion. Cellular PKC activity was monitored by assay of the

expression of the PKC-regulated gene *egr-1* at different concentrations of PMA at 24 hours. Results from this study are plotted in Figure 2. Addition of 100 nM of PMA for 24 hours induced *egr-1* expression in Jurkat and activated *ras* containing cells, demonstrating chronic stimulation of PKC activity by PMA at this concentration. Exposure to a higher dose of PMA (500 nM) for 24 hours inhibited the *egr-1* gene expression in both cell types, demonstrating down-regulation of PKC activity.

Down-regulation of intracellular protein kinase C activity by 24 hours of treatment with PMA resulted in cessation of proliferation in the control Jurkat cells. Following replating, exponential proliferation resumed after a 48 hours delay period (Figure 2). Very different growth and survival characteristics were observed in the activated *ras* containing cells following down-regulation of protein kinase C. After 24 hour treatment with PMA, the number of activated *ras* containing cells surviving down-regulation of PKC decreased over three days. No recovery or regrowth was subsequently observed.

Example 6 Decreased Survival of Cells Transfected with Activated *ras*.

To determine whether the decrease in survival of activated *ras* containing cells after down-regulation of PKC was due to apoptosis, the relative degree of nuclear DNA fragmentation, a characteristic feature of apoptosis, was determined. A portion of the cells shown in Figures 1 and 2 were stained with propidium iodide and analyzed on a fluorescence activated cytometer (Figure 3). After down-regulation of PKC, the percentage of nuclear DNA fragmentation in activated *ras* containing cells increased 4-fold, while in control Jurkat cells no significant increase in DNA fragmentation was observed. This marked increase in nuclear DNA fragmentation was observed in the activated *ras* containing cells at multiple time points over days following down-regulation of PKC, correlating with the decreases in activated *ras* containing cell number over the three days (Figure 2). Cell cycle kinetics after down-regulation of PKC were analyzed by

examination of DNA profiles of the same populations of Jurkat and activated *ras* containing cells studied in Figures 1 and 2 (Figure 4). After down-regulation of PKC, the majority of Jurkat cells accumulated in the G_0/G_1 (2n) phase. In contrast, no such G_1 cell cycle arrest occurred in the activated *ras* containing cells, and those cells remained distributed throughout the cell cycle.

As independent confirmation of the requirement for a decrease in PKC activity for induction of p21*ras*-activated apoptosis, an inhibitor of PKC, staurosporin, was utilized in parallel experiments. Staurosporin treatment activated apoptosis in activated *ras* containing cells with faster kinetics than did chronic exposure to high-dose PMA, as might be expected from the more rapid action of staurosporin on inhibition of PKC. Twelve hours after exposure to staurosporin, over 26% of activated *ras* containing cells demonstrated significant nuclear DNA fragmentation, whereas only 8% of the parental Jurkat cells exhibited this indicator of programmed cell death. A one hour exposure to staurosporin resulted in a 55% decrease in the number of viable activated *ras* containing cells enumerated 3 days later, while the same treatment did not decrease the number of Jurkat cells. Thus, a transient suppression of PKC activity causes a significant increase in cell death in *ras*-activated cells relative to non-activated cells and the cell deaths are due to apoptosis.

Example 7 *Bcl-2* Prevents Apoptosis by Activated p21*ras*.

A human *bcl-2* gene was introduced into Jurkat cells (Jurkat/*bcl-2*) and double transfected (activated *ras*/*bcl-2*) cells made to investigate the effect of *bcl-2* over expression on the cell death induced by activated p21*ras* after inhibition of PKC activity. The growth curves of the two *bcl-2* transfected cell lines were similar to the untransfected parental cell lines under normal growth conditions (Figure 1). Over expressed *bcl-2* dramatically inhibited the apoptosis induced by constitutively-activated p21*ras* after down-regulation of PKC (Figures 2 and 3). At 24 hours and 48 hours after down-regulation of PKC, the

majority of activated *ras* and *bcl-2* transfected cells were arrested in the G_1 phase of cell cycle (Figure 4), like the control (parental) Jurkat cells. As the PKC activity was restored, the activated *ras/bcl-2* cells resumed proliferation (Figure 2). Over expressed *bcl-2* thus blocked both p21*ras*-induced apoptosis as well as *Ras*-induced cell cycle progression in the setting of PKC down-regulation. *bcl-2* had no demonstrable effect on cell growth kinetics in the absence of co-expression of activated p21*ras*. Jurkat cells transfected with *bcl-2* were similar to untransfected Jurkat cells in terms of growth rate, extent of nuclear DNA fragmentation, and cell cycle progression in the presence or absence of PKC activity.

Example 8 <u>*Ras*-Dependent *In Vivo* Phosphorylation of *Bcl-2*</u>.

To assess the phosphorylation state of Bcl-2 protein in the setting of stimuli promoting *ras*-induced apoptosis, cells were metabolically-labeled with $^{32}P-PO_4$ under normal conditions or after down-regulation of PKC activity. Bcl-2 protein was immunoprecipitated using an anti-Bcl-2 antibody and resolved on denaturing gels (Figure 5). Simultaneously, a fraction of the total lysate from each cell type was also resolve by electrophoresis to verify successful and equivalent labeling of proteins in each cell type. Bcl-2 protein (26 kDa) was phosphorylated in activated *ras* and *bcl-2* transfected cells after the suppression of PKC activity. Several other phosphate-labeled bands were also precipitated by anti-Bcl-2 antibody. These proteins may be antigenically related to Bcl-2 as they appeared to immunoprecipitate with another anti-Bcl-2 antibody preparation. Alternatively, they may instead represent specific binding of Bcl-2 to other phosphoproteins. The level of Bcl-2 protein expression during normal growth and after down-regulation of PKC was studied by immunoprecipitation and immunoblotting with an anti-Bcl-2 antibody (Figure 5). The 24 kDa Bcl-2 protein was apparent in cells transfected with the *bcl-2* gene, both under normal growth conditions and after down-regulation of PKC activity, and there was no

significant change in the level of Bcl-2 protein expression under either circumstance. Thus, Bcl-2 protein in phosphorylated in response to the change in the intracellular environment engendered by activated p21*ras* and the suppression of the PKC activity.

Example 9 Ras-Dependent *In Vitro* Phosphorylation of *Bcl-2*.

Phosphorylation of Bcl-2 protein *in vitro* was studied to determine relative kinase activities on Bcl-2 in response to PKC down-regulation in the presence of activated p21*ras*. The 26 kDa phosphorylated Bcl-2 protein was detected in immune complexes at increased levels only from permeabilized activated *ras* and *bcl-2* transfected cells and only after down-regulation of PKC activity (Figure 5, lane 5). A 21 kDa newly-kinased protein species was also detected under these conditions, possibly representing the Bcl-2 partner, Bax. The appearance of this labeled protein in the co-precipitations after *in vitro*, but not *in vivo* labeling may reflect differences in the turnover of this labeled species of intrinsic differences in the two experimental techniques. Phosphorylation of these proteins was inhibited by the serine threonine kinase inhibitor, staurosporin, which suggests the involvement of a serine threonine kinase in Bcl-2 phosphorylation during stimulation of *ras*-induced apoptosis. In contrast, the tyrosine protein kinase inhibitor genistein had no effect on the phosphorylation of Bcl-2 in this permeabilzed cell system. To confirm the identity of the phosphorylated band as Bcl-2 immunoprecipitation was conducted, after [^{32}P]ATP labeling, using anti-Bcl-2 antibody. Precipitated products were separated and transferred to nitrocellulose for autoradiography and immunoblotting. Results revealed that the labeled 26 kDa protein was immunoreactive with Bcl-2 antibody (Figure 5, lanes 1 and 2).

Example 10 Induction of Apoptosis by Activated p21*ras*.

Jurkat cell lines expressing varying amounts of v-Ha-*ras* were examined (Figure 7) to assess the correlation between the extent of the apoptosis

induced by activated *ras* after PKC down-regulation and the levels of the *ras* expression. Clones 6, 7, 19 and 20 expressed progressively higher levels of *ras* mRNA, with the highest-expressing clone (clone 7) expressing two-fold more *ras* than the lowest expressing clone (clone 20). Each clone was assayed for nuclear DNA fragmentation by flow cytometry after down-regulation of PKC (Figure 8). Clones 6 and 20, which express lower levels of *ras* mRNA, had lower percentages of v-*ras*-induced nuclear DNA fragmentation (20-25%). In contrast, clones 7 and 19 demonstrated greater than 40% DNA fragmentation (Figure 8). These data suggest that the extent of the apoptosis induced by activated p21*ras* is relative to the level of *ras* expression, and indicates that apoptosis observed is due to the *ras* gene product.

Example 11 <u>Protein Synthesis Required for Apoptosis Induced by Activated p21*ras*</u>.

Completion of pathways leading to nuclear DNA fragmentation and apoptosis are in some cases dependent upon new synthesis of macromolecules. The dependence of the DNA fragmentation observed after down-regulation of protein kinase C in the presence of activated *ras* genes was determined by exposure of the cells to the protein synthesis inhibitor cycloheximide at a concentration that inhibited the incorporation of [^{35}S]-methionine and [^{35}S]-cysteine into new protein by >90%. This protein synthesis inhibitor was added to the cell lines after down regulation of PKC, and nuclear DNA fragmentation was analyzed. The percentage of nuclear DNA fragmentation in activated *ras* containing cells decreased from 38% to 24% when cycloheximide was present for the last 6 hours of PKC down-regulation (Figure 9). In parental Jurkat cells, the percentage of nuclear DNA fragmentation after down-regulation of PKC was also decreased, but to a lesser degree. Thus, the maximal induction of apoptosis by down-regulation of protein kinase C in the setting of activated p21*ras* was dependent upon new protein synthesis.

Example 12 *Ras*-Induced Pathway for Apoptosis is Partially Calcium-Dependent.

Apoptotic pathways leading to nuclear DNA fragmentation in lymphocytes can be active by signals which result in calcium mobilization, either from intracellular pools or from extracellular sources. Because an activated *ras* gene has been reported to alter calcium flux in lymphocytes, the effects of calcium ionophore on the growth and viability of these cell lines were examined. Calcium ionophore (at a final concentration of 2 μM) was added to Jurkat and activated *ras* transfected cell cultures. Treated cells were assayed for cell growth kinetics and nuclear DNA fragmentation (Figure 10). Growth curves of both cell lines in the presence of the calcium ionophore were similar. Survival decreased rapidly over the first two days in both cell types. Subsequently, both cell types retained the ability to proliferate which may be due to the decay of the calcium ionophore (Figure 11). The percentage of nuclear DNA fragmentation was examined in parallel experiments. Exposure to calcium ionophore increased nuclear DNA fragmentation to equivalent degrees in both cell lines, comparable to the effects on cell growth (Figure 12).

EGTA, a chelator of extracellular calcium, prevented the induction of nuclear DNA fragmentation by the ionophore, demonstrating that the action of the ionophore was dependent on extracellular calcium (Figure 12). EGTA was used to determine if the *ras*-induced apoptosis in activated *ras* containing cells was dependent on extracellular calcium. EGTA moderately reduced the percentage of DNA fragmentation in activated *ras* containing cells when added during the last 6 hours of PKC down regulation (Figure 10), but not to the same extent as it inhibited ionophore-induced nuclear DNA fragmentation (Figure 12). Over expression of the *bcl-2* gene also was consistently protective against the induction of nuclear DNA fragmentation by calcium ionophore in both normal Jurkat cells and activated *ras* containing cells (Figure 12). A

calcium chelator is thus unable to completely prevent apoptosis due to PKC inhibition in cells with an activated *ras* oncogene. The mechanism by which PKC induces apoptosis in *ras* neoplasms overlaps, but does not totally encompass the simple dysregulation of calcium.

Example 13 Activated *ras* under Conditions of Serum-Deprivation.

Jurkat and activated *ras* containing cells were cultured in medium containing 0.05% serum to determine the influence of serum depletion on *ras*-induced apoptosis. DNA fragmentation was assayed for 4 consecutive days (Figure 13). Nuclear DNA fragmentation increased with time in both cell types. Activated *ras* containing cells exhibited slightly higher levels of nuclear DNA fragmentation without treatment (control) and at subsequent time points compared to parental Jurkat cells, but there was no significant effect of activated p21*ras* on apoptosis during serum-deprivation relative to controls.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. The specification and examples should be considered exemplary only with the true scope and spirit of the invention indicated by the following claims.

I Claim:

1. A pharmaceutical composition comprising an agent that induces apoptosis of aberrantly proliferating cells that express an activated oncogene.

2. The composition of claim 1 wherein the agent is calphostin C, chelerythrine chloride, H7, hexdecyl-methylglycerol, hexadecyl-acetylglycerol, hypericin, K252, RO318220, phloretin, bryostatin, sphingosine, staurosporin or derivatives or combinations thereof.

3. The composition of claim 1 wherein the activated oncogene is a member of the *ras* gene family.

4. The composition of claim 1 wherein the agent reduces intracellular protein kinase activity.

5. The composition of claim 4 wherein the intracellular protein kinase activity is protein kinase C activity.

6. The composition of claim 1 further comprising a pharmaceutically acceptable carrier selected from the group consisting of water, alcohols, oils, salts, saccharides, starches, fatty acids, buffers, stabilizers, anti-oxidants, preservatives and combinations thereof.

7. The composition of claim 1 further comprising an other agent that increases cellular calcium concentrations.

8. The composition of claim 7 wherein the other agent is a calcium ionophore selected from the group consisting of thapsigargin, ionomycin, A23187 and derivatives and combinations thereof.

9. A pharmaceutical composition comprising an agent that regulates cellular calcium concentrations and induces apoptosis of aberrantly proliferating cells that express an activated oncogene.

10. A method for inducing apoptosis of aberrantly proliferating cells by administering an agent that suppresses cellular protein kinase activity wherein

said cells express an activated oncogene.

11. The method of claim 10 wherein induction of apoptosis is characterized by an increase of nuclear DNA fragmentation.

12. The method of claim 11 wherein the nuclear DNA fragmentation increase is between about two fold and about ten fold.

13. The method of claim 10 wherein the aberrantly proliferating cells are human neoplastic cells.

14. The method of claim 13 wherein the neoplastic cells are malignant.

15. The method of claim 10 wherein the aberrantly proliferating cells are infected human cells.

16. The method of claim 15 wherein the infected human cells are virally infected.

17. The method of claim 10 wherein the agent is calphostin C, chelerythrine chloride, H7, hexdecyl-methylglycerol, hexadecyl-acetylglycerol, hypericin, K252, RO318220, phloretin, bryostatin, sphingosine, staurosporin, thapsigargin, ionomycin, A23187 or derivatives or combinations thereof.

18. The method of claim 10 wherein the cellular protein kinase activity is protein kinase C activity.

19. The method of claim 10 wherein the activated oncogene is a member of the *ras* gene family.

20. The method of claim 10 wherein the activated oncogene expresses p21*ras*.

21. The method of claim 10 wherein the aberrantly proliferating cells are transfected with said activated oncogene.

22. A method for treating a cell proliferative disorder of a patient comprising the step of administering a therapeutically effective amount of an agent that reduces intracellular protein kinase activity.

23. The method of claim 22 wherein the cell proliferative disorder is a neoplasm or an infection.

24. The method of claim 23 wherein the neoplasm is selected from the group consisting of lymphomas, sarcomas, carcinomas, glioblastomas, leukemias, myelodysplastic disorders, virally-induced cancers, carcinomas, melanomas and neuromas.

25. The method of claim 23 wherein the infection is a viral infection.

26. The method of claim 22 wherein the patient is a human.

27. The method of claim 22 wherein the therapeutically effective amount is that amount which reduces intracellular protein kinase activity sufficiently to induce apoptosis.

28. The method of claim 22 wherein the agent comprises diacylglycerol, monoacylglycerol, or derivatives or functional equivalents thereof.

29. The method of claim 28 wherein the functional equivalent is 1-oleoyl-2-acetylglycerol, diaoctanoylglycerol,

30. The method of claim 22 wherein the agent comprises an anti-sense nucleic acid.

31. The method of claim 22 wherein the agent is staurosporine, bryostatin or RO318220.

32. The method of claim 22 further comprising the step of administering a calcium ionophore to the patient.

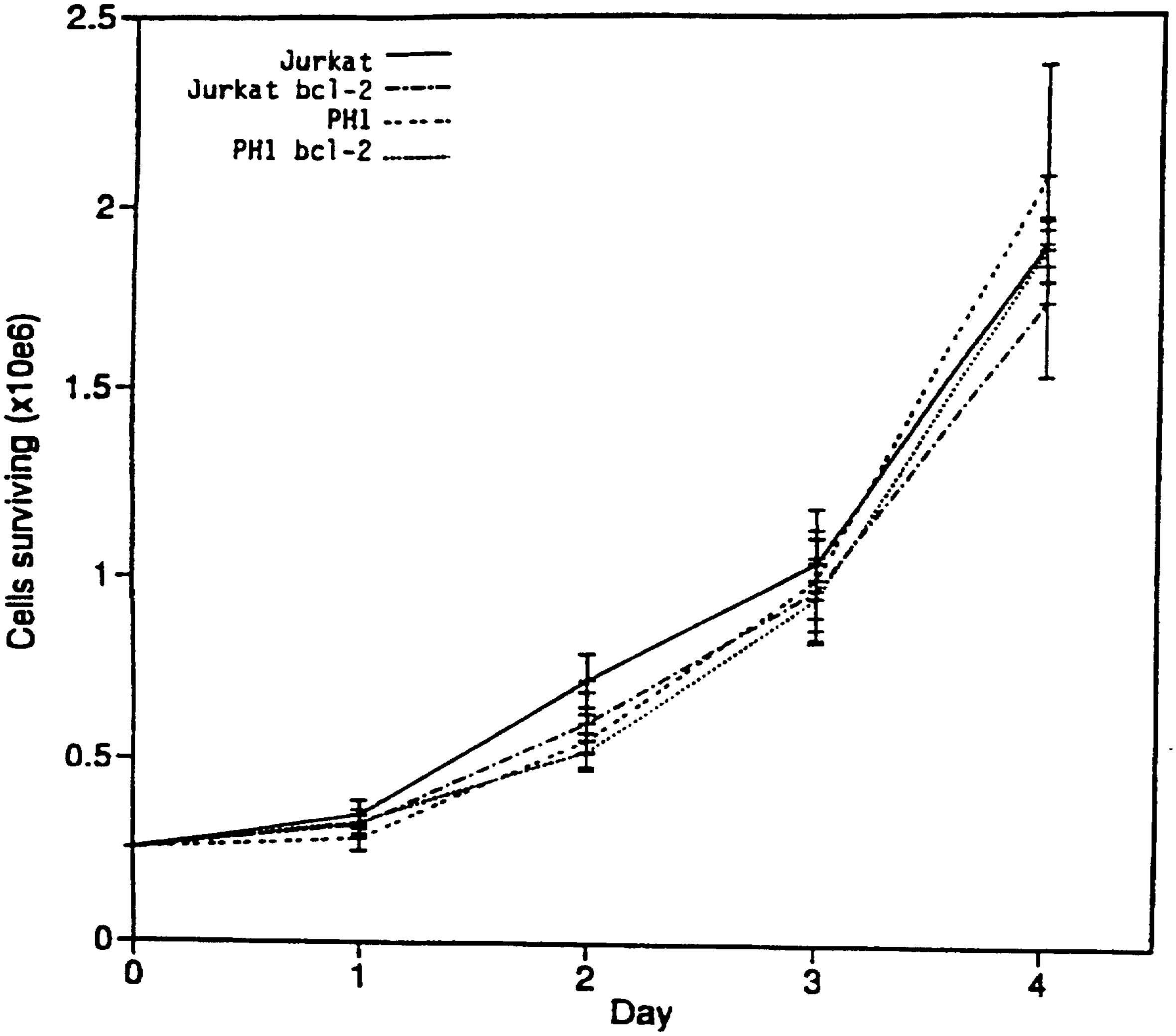
33. The method of claim 32 wherein the calcium ionophore is selected from the group consisting of calcium, A23187, thapsigargin and ionomycin.

34. The method of claim 22 wherein the neoplastic disorder is a benign or malignant tumor.

35. The method of claim 34 wherein the neoplastic disorder is a carcinoma, sarcoma, lymphosarcoma or leukemia.

36. The method of claim 34 wherein the neoplastic disorder comprises cells

with an activated *ras* gene.



2.5
2
1.5
1
0.5
0
Cells surviving (x10e6)
Jurkat
Jurkat bcl-2
PH1
PH1 bcl-2
0
1
2
3
4
Day

FIG. 1

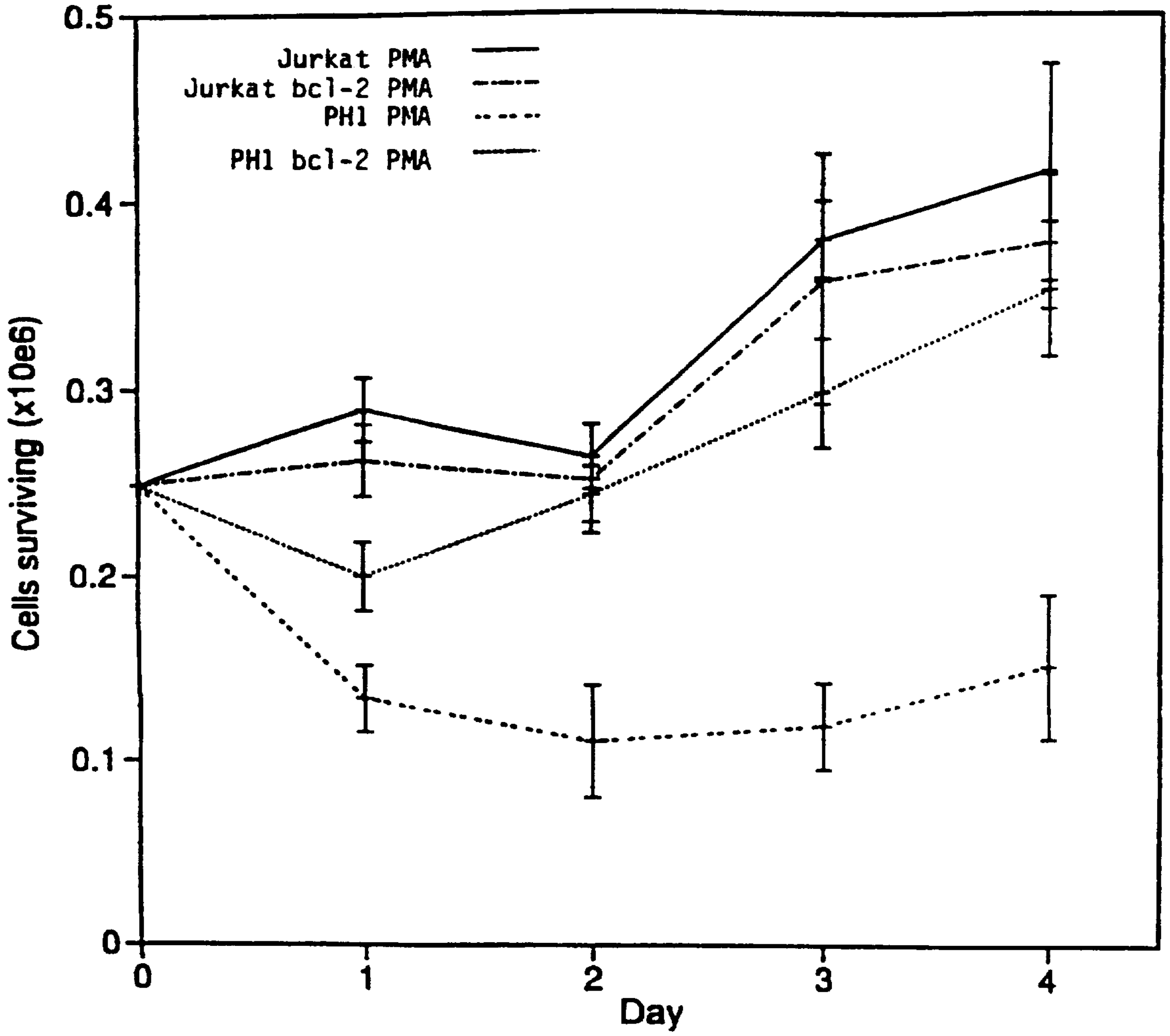

Jurkat PMA
Jurkat bcl-2 PMA
PH1 PMA
PH1 bcl-2 PMA
Cells surviving (x10e6)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
Day

FIG. 2

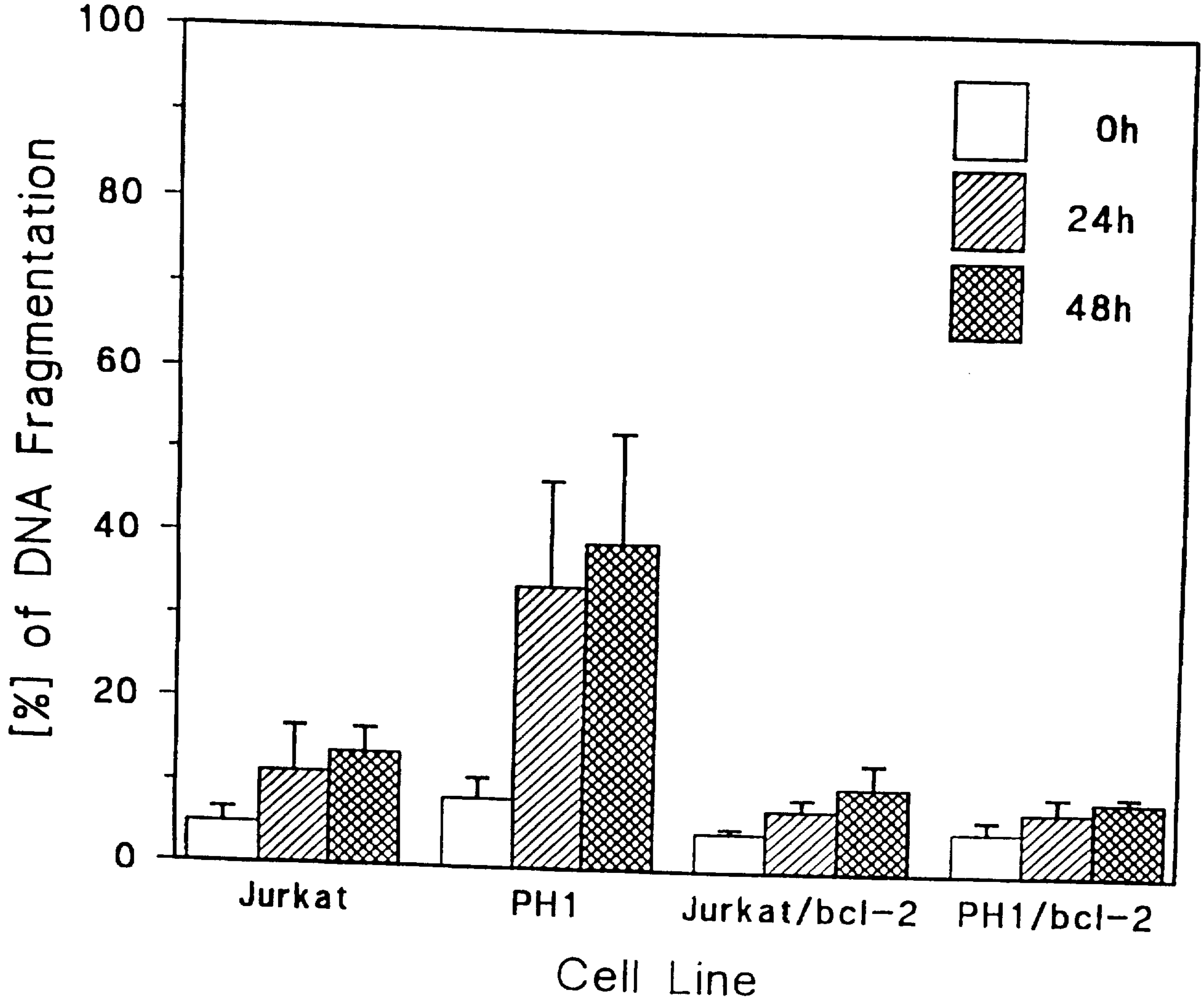

100
80
60
40
20
0
[%] of DNA Fragmentation
0h
24h
48h
Jurkat
PH1
Jurkat/bcl-2
PH1/bcl-2
Cell Line

FIG. 3

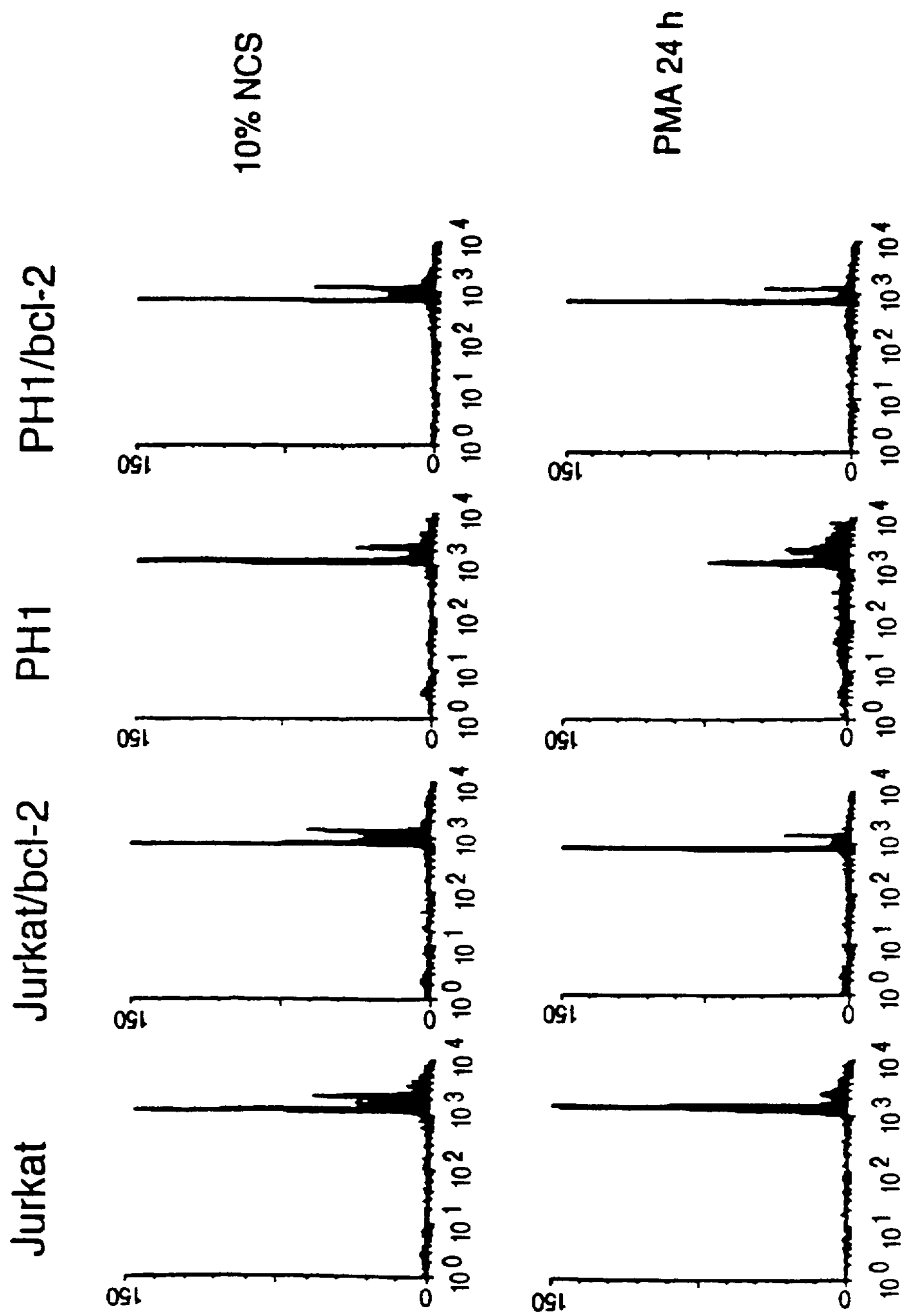

Jurkat
Jurkat/bcl-2
PH1
PH1/bcl-2
10% NCS
PMA 24 h
150
0
10^0 10^1 10^2 10^3 10^4

FIG. 4

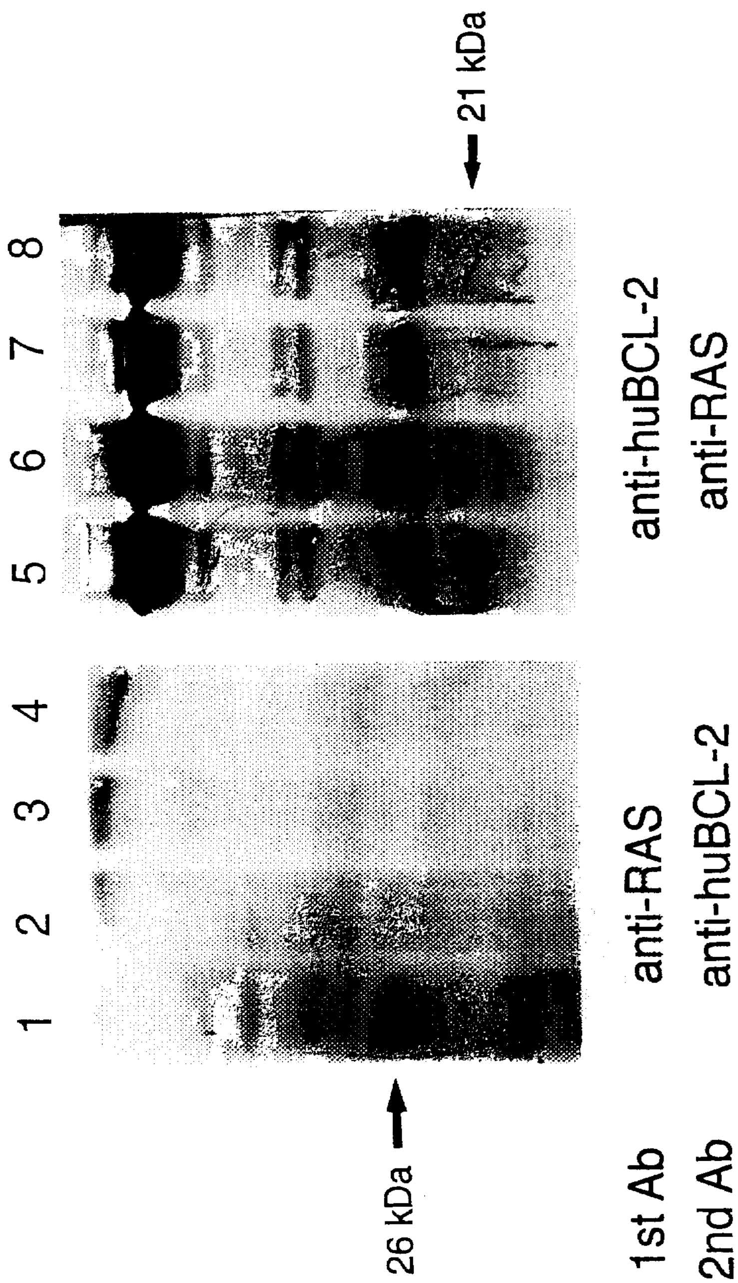

21 kDa
26 kDa
1 2 3 4 5 6 7 8
1st Ab anti-RAS anti-huBCL-2
2nd Ab anti-huBCL-2 anti-RAS

FIG. 5

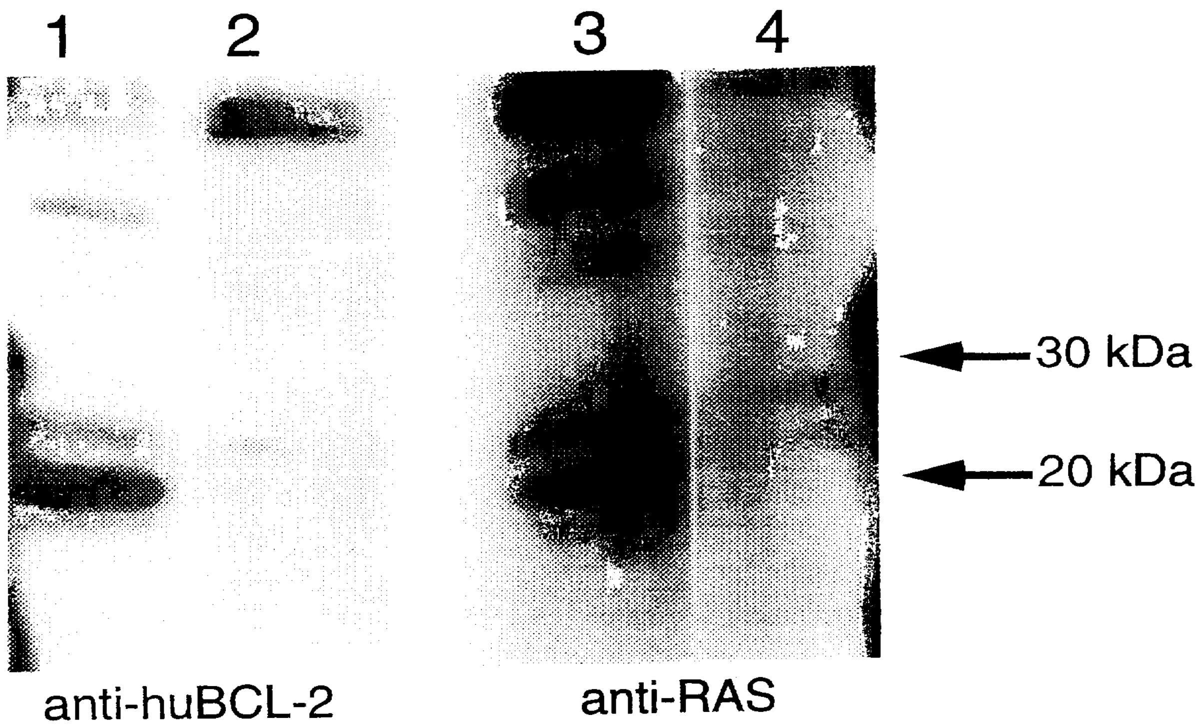


FIG. 6

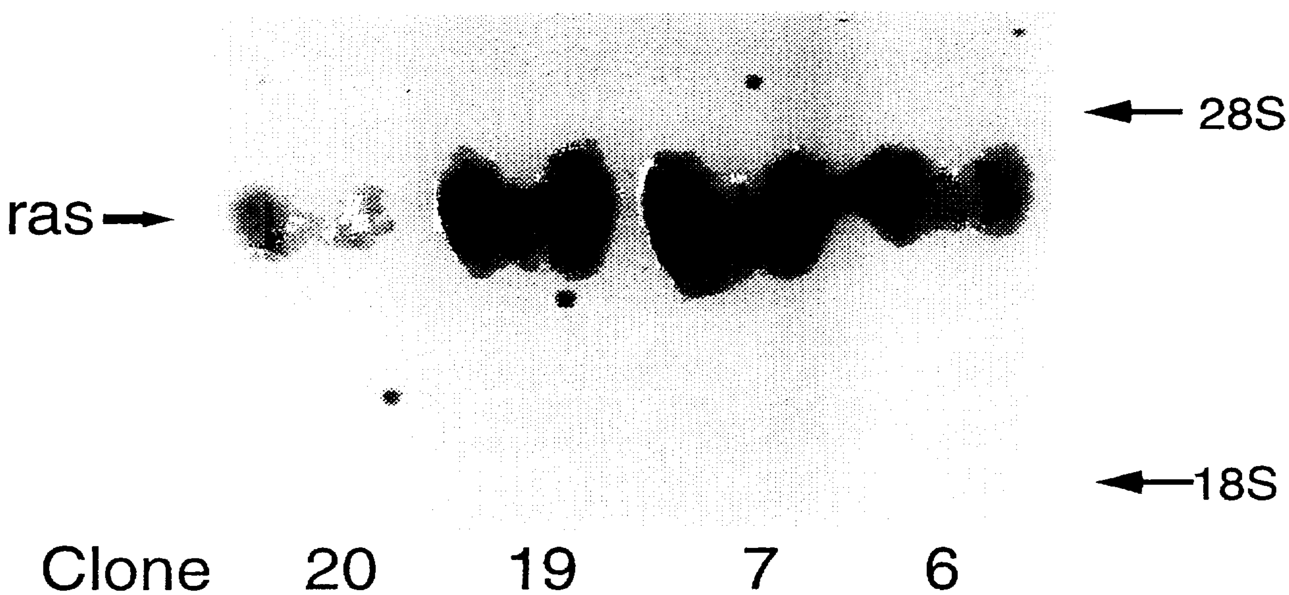


FIG. 7

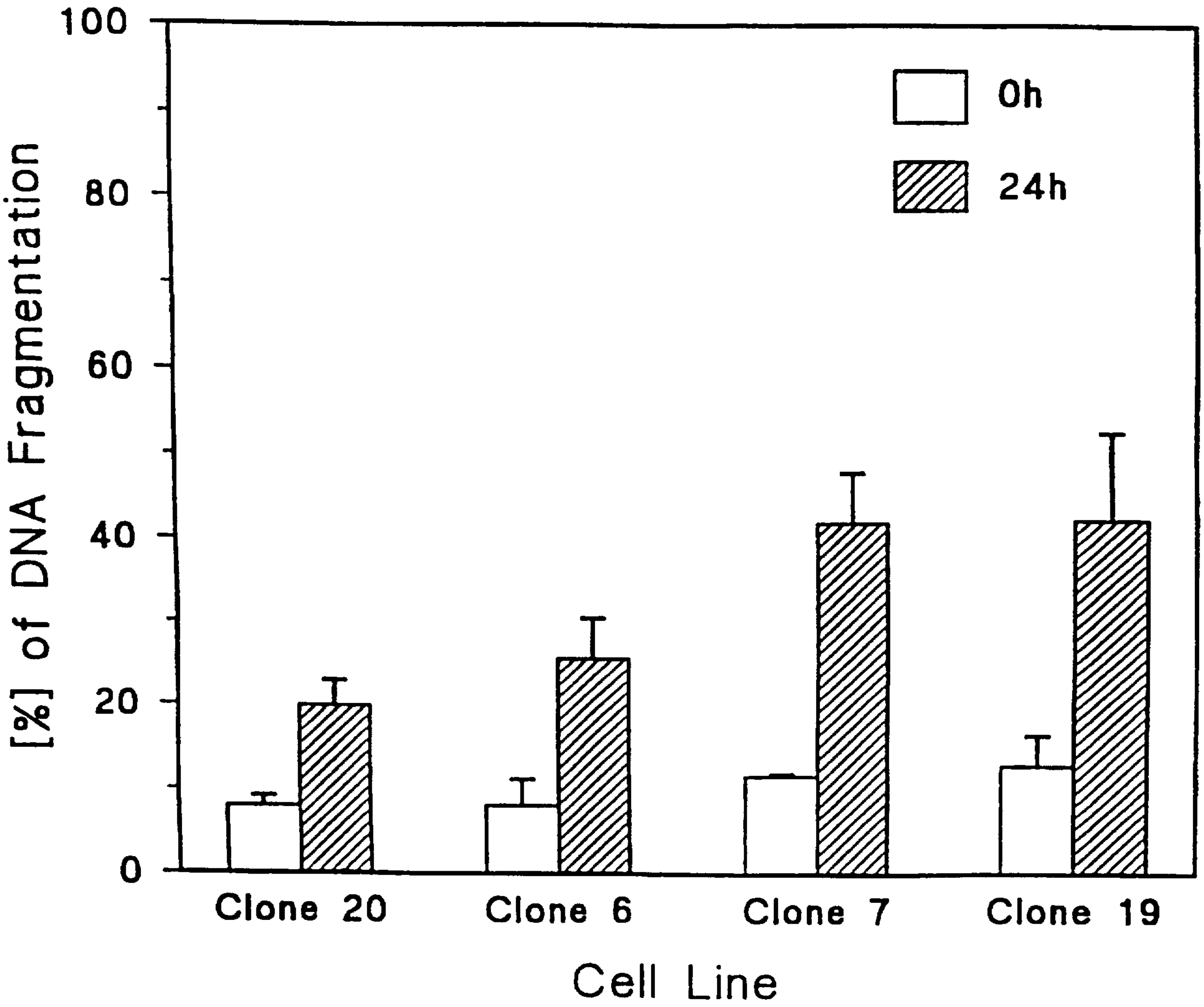

100
80
60
40
20
0
[%] of DNA Fragmentation
0h
24h
Clone 20
Clone 6
Clone 7
Clone 19
Cell Line

FIG. 8

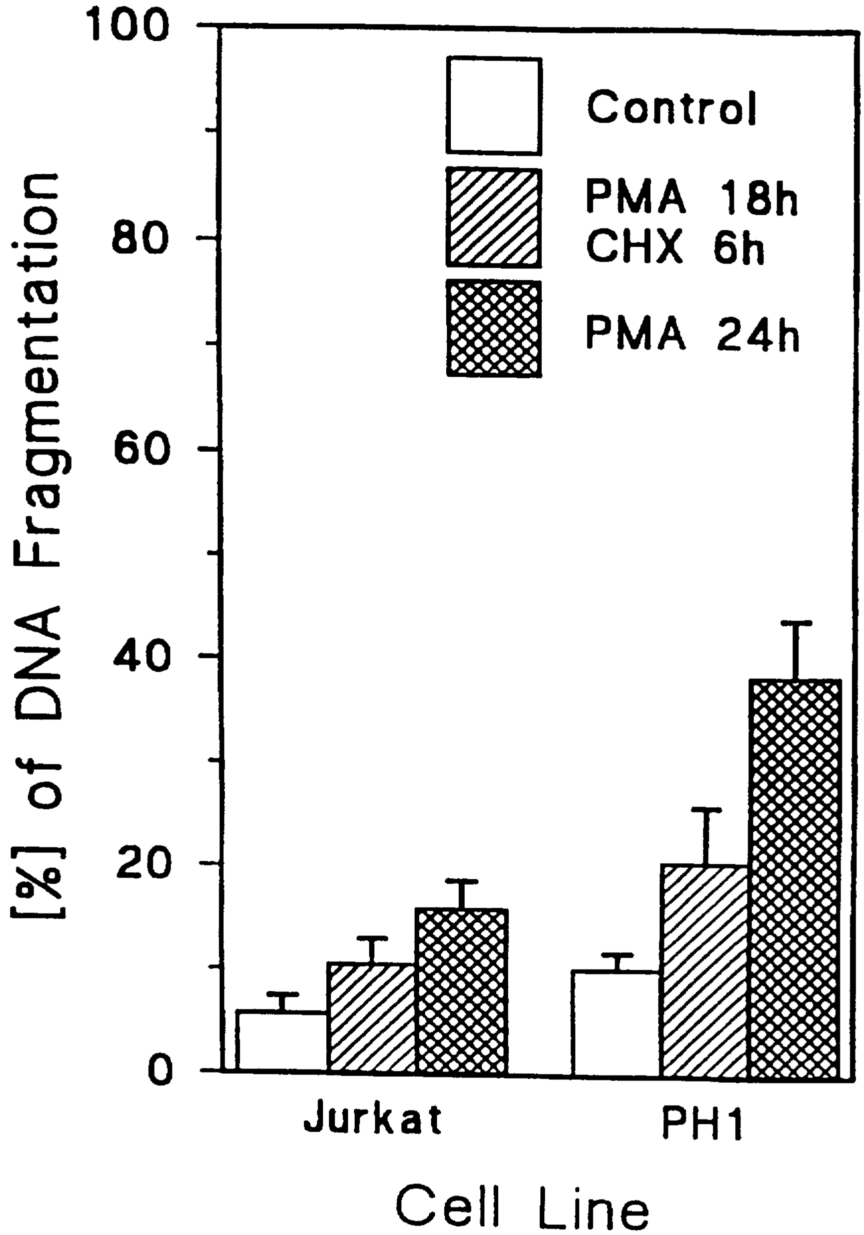

100
80
60
40
20
0
[%] of DNA Fragmentation
Control
PMA 18h
CHX 6h
PMA 24h
Jurkat
PH1
Cell Line

FIG. 9

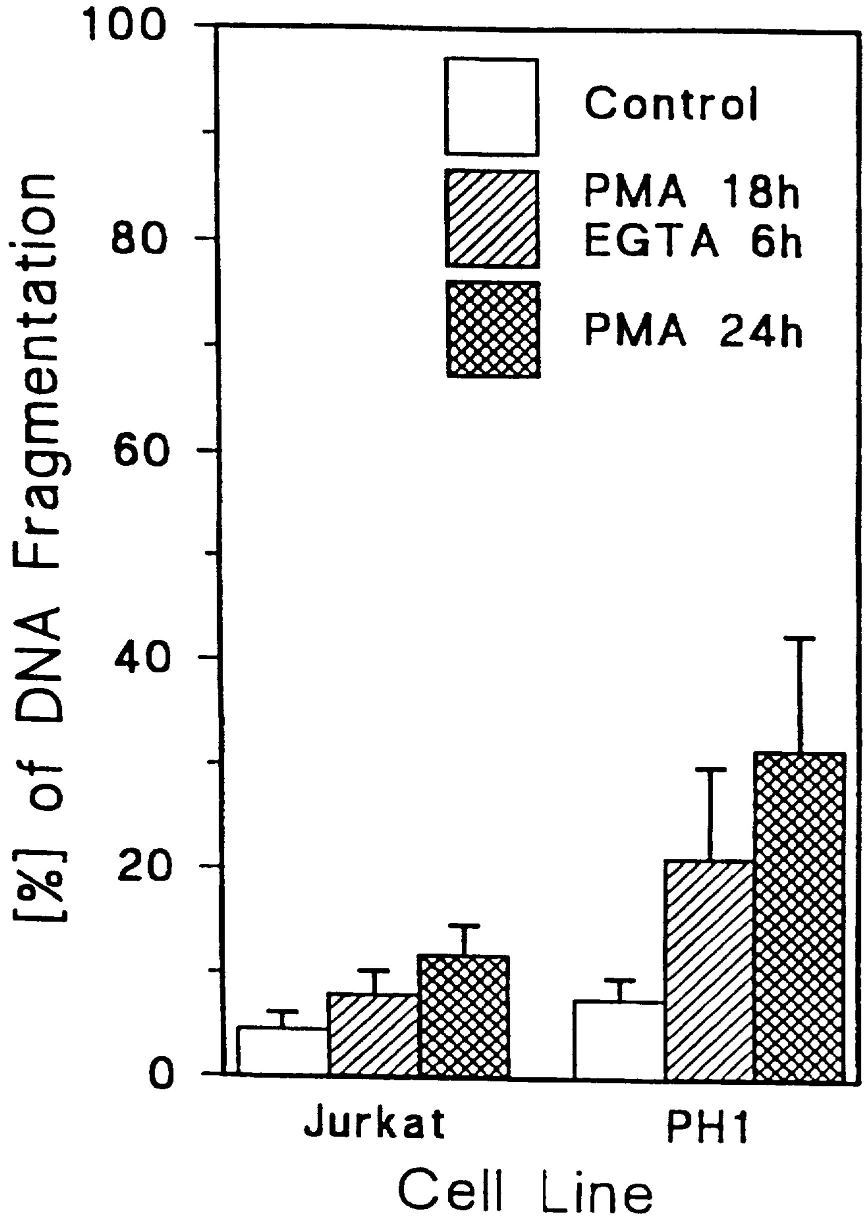

100
80
60
40
20
0
[%] of DNA Fragmentation
Control
PMA 18h
EGTA 6h
PMA 24h
Jurkat
PH1
Cell Line

FIG. 10

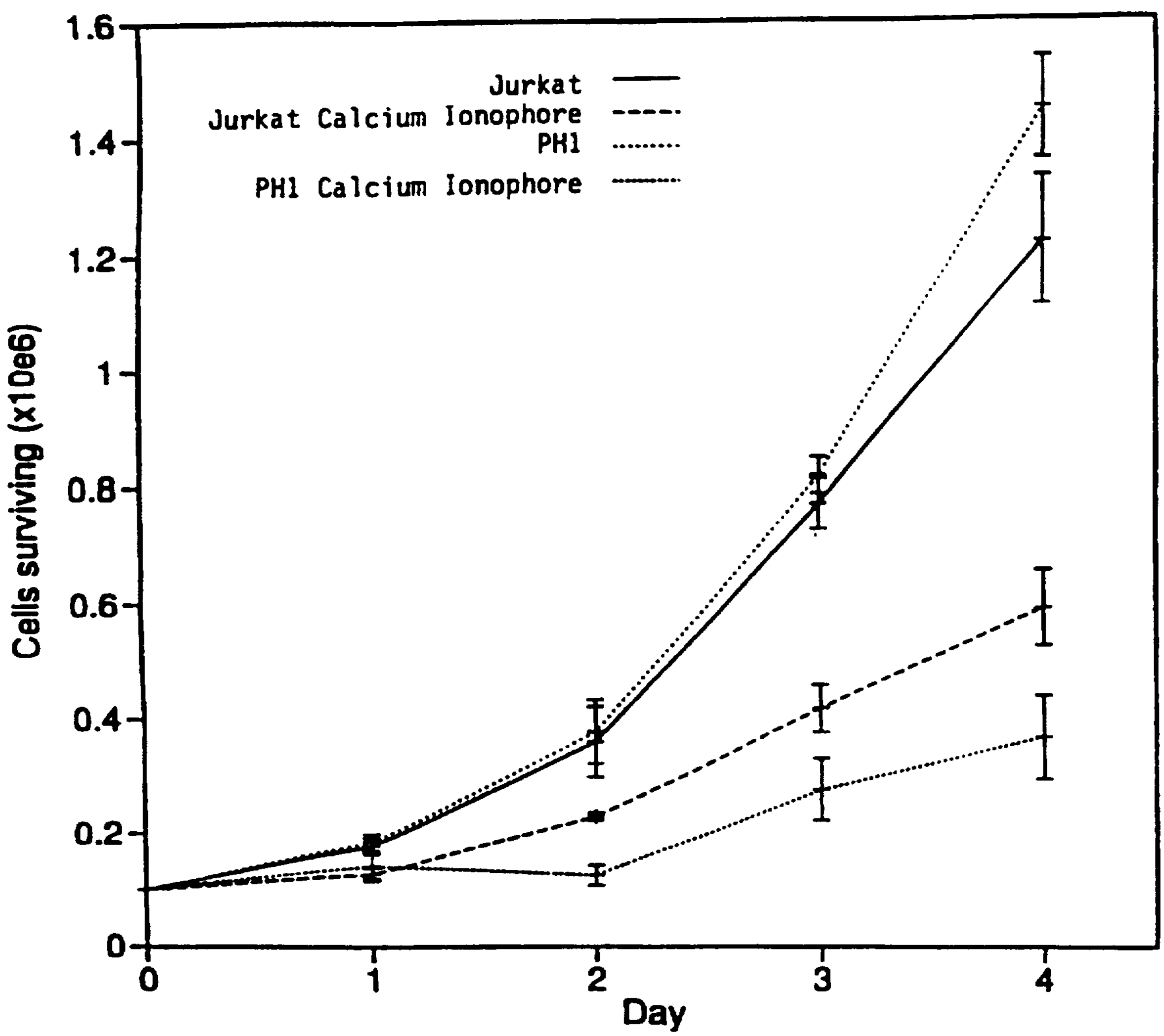

Jurkat
Jurkat Calcium Ionophore
PH1
PH1 Calcium Ionophore
Cells surviving (x10e6)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Day
0
1
2
3
4

FIG. 11

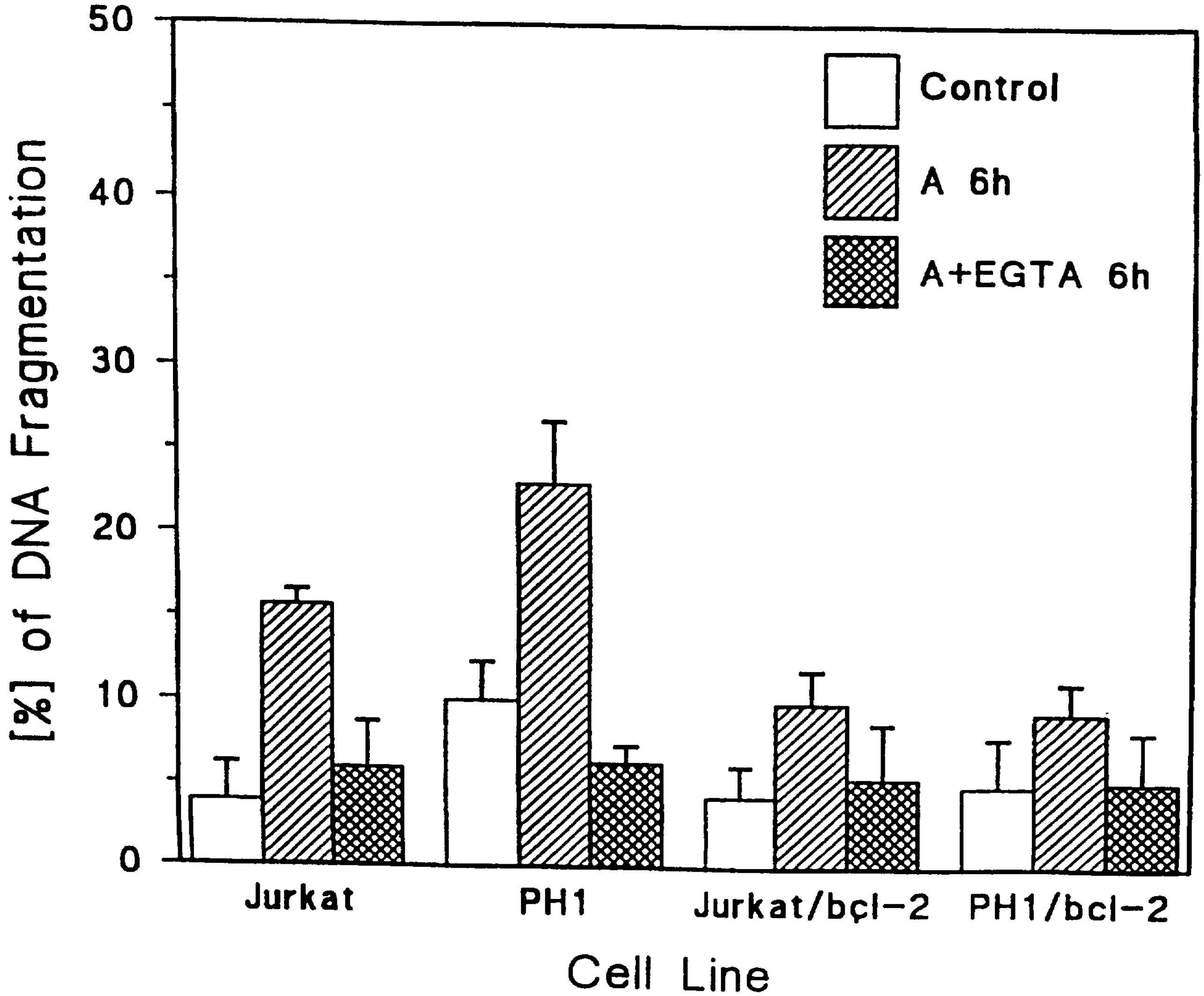

50
40
30
20
10
0
[%] of DNA Fragmentation
Control
A 6h
A+EGTA 6h
Jurkat
PH1
Jurkat/bcl-2
PH1/bcl-2
Cell Line

FIG. 12

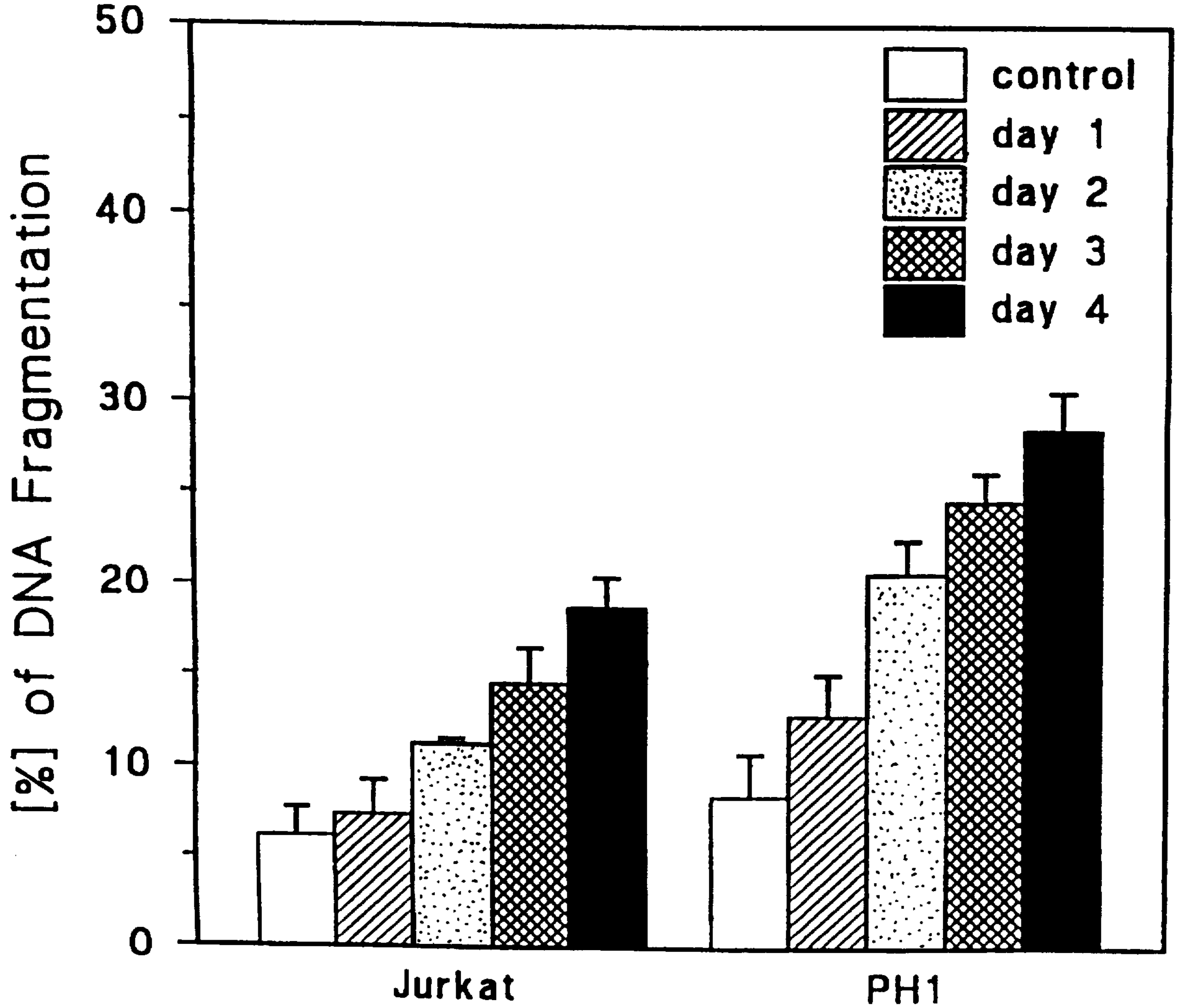

50
40
30
20
10
0
[%] of DNA Fragmentation
control
day 1
day 2
day 3
day 4
Jurkat
PH1
Cell Line

FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/12381

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/00 A61K31/12 A61K31/13 A61K31/215 A61K31/23
A61K31/35 A61K31/40 A61K31/47 A61K31/495

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEUK. RES., vol. 19, no. 9, 1995, pages 667-673, XP000610068 R.M. MOHAMMAD ET AL: "Bryostatin I induces apoptosis and augments inhibitory effects of vincristine in human diffuse large cell lymphoma." * abstract * ---	1,2,4-6, 10,11, 13,14, 17,18, 22-24, 31,34
X	DATABASE WPI Week 8543 Derwent Publications Ltd., London, GB; AN 85-266688 XP002020124 & JP,A,60 178 815 (RIKAGAKU) , 12 September 1985 see abstract ---	1,2

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
3 December 1996	17.12.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Klaver, T

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 8733 Derwent Publications Ltd., London, GB; AN 87-231627 XP002020125 & JP,A,62 155 284 (KYOWA) , 10 July 1987 see abstract ---	1,2
X	US,A,4 816 462 (NOWICKY) 28 March 1989 see column 1 - column 2 ---	1,2
X	FEBS LETT., vol. 367, no. 3, 1995, pages 301-305, XP002020122 S. ALALUF ET AL.: "Rapid agonist mediated phosphorylation of the metabotropic glutamate receptor 1a by protein kinase C in permanently transfected BHK cells."	1,2
X	& DATABASE MEDLINE knight-ridder # 95331396 , S.ALALUF ET AL: see abstract ---	1,2
X	CANCER RES., vol. 55, no. 3, 1995, pages 691-697, XP000611413 H. OTHA ET AL.: "Induction of apoptosis by sphingosine in human leukemic HL-60 cells. a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation." see the whole document ---	1,2,4-6, 10-14, 17,18, 22-24, 26,27, 31,34,35
X	AM. J. PATHOL., vol. 145, no. 6, 1994, pages 1265-1270, XP000610087 D. LESZCZYNSKI ET AL.: "Apoptosis of vascular smooth muscle cells. Protein kinase C and oncoprotein Bcl-2 are involved in regulation of apoptosis in non-transformed rat vascular smooth muscle cells." abstract ---	1,2,4-6, 10,17, 18,22,26
X	J. NEUROCHEM., vol. 62, no. 2, 1994, pages 479-488, XP002020123 P. KAHLE ET AL.: "Protein kinase inhibitor H-7 differentially affects early and delayed nerve growth factor responses in PC12 cells." see the whole document --- -/--	1,2,4-6

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHIM BIOPHYS. ACTA, vol. 1154, no. 3-4, 1993, pages 223-236, XP000610062 Y.A. HANNUN ET AL.: "Sphingolipid breakdown products: anti-proliferative and tumor-suppressor lipids." -----	

1

INTERNATIONAL SEARCH REPORT

I national application No.

PCT/US 96/ 12381

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 22-36
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 96/12381

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4816462	28-03-89	AU-B-	566682	29-10-87
		AU-A-	8597782	24-02-83
		PT-A-	75226	25-05-83
		US-A-	4970212	13-11-90